THE ROLE OF PULMONARY INTRAVASCULAR MACROPHAGES IN THE DEVELOPMENT OF HEAVES IN HORSES

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the Department of Veterinary Biomedical Sciences

University of Saskatchewan

Saskatoon

By

Karin Aharonson-Raz

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervise my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Veterinary Biomedical Sciences
Western College of Veterinary Medicine
52 Campus Drive
University of Saskatchewan
Saskatoon, Saskatchewan
S7N 5B4 Canada

ABSTRACT

Heaves is triggered by exposure to dust and its components, such as endotoxin, and is characterized by clinical signs such as coughing, decreased exercise tolerance, difficulty breathing and abnormal lung sounds which are due to bronchoconstriction and accumulation of neutrophils in the airways. Pulmonary intravascular macrophages (PIMs) are believed to increase horses' sensitivity to endotoxemia-induced lung inflammation. The first objective of this study was to investigate a hitherto unknown role of PIMs in equine heaves. I used mouldy hay (MH) to induce heaves and gadolinium chloride (GC) to deplete PIMs in order to compare responses between non-treated and GC-treated heaves horses. A modified randomized crossover study (2X2 factorial) was conducted in which mares (N=9) were exposed to 4 different treatments: alfalfa cubes (Cb), alfalfa cubes + GC (Cb-GC), mouldy hay (MH) and MH + GC (MH-GC). Each treatment was followed by broncholaveolar lavage (BAL). MH was fed for 7 days to induce heaves followed by Cb for 21 days to achieve remission, whereas the treatments in which heaves was not induced (Cb; Cb-GC), the cubes were fed prior to the BAL and for 14 days after the BAL to allow recovery from the BAL procedure. BAL fluids were processed to investigate total cell, neutrophil and alveolar macrophage concentrations. In addition, TNFα protein levels as well as TNFα, IL-8, and TLR4 mRNA expression in BAL cells were assessed in order to infer on their activation state.

Data showed higher concentration of dust (3X), endotoxin (20X), and endotoxin per milligram of dust (7X) in MH compared to the Cb environment. Clinical scores and neutrophil concentrations in BAL were higher when mares received MH compared to MH and GC (MH-GC). Real time reverse transcriptase PCR revealed a significant lower

expression of IL-8 and TLR4 mRNA in BAL cells from MH-GC mares compared to MH. TNFα mRNA expression as well as protein concentration were not affected by the different treatments. *In vitro* secondary LPS challenge significantly increased IL-8 mRNA expression in cells from MH treatment compared to without LPS, but not in the MH-GC treatment. TLR4 expression was not affected by the secondary challenge. Although secondary LPS challenge increased expression of TNFα mRNA and protein, the differences among treatment groups were not meaningful. In conclusion, PIM depletion attenuates clinical scores, migration of inflammatory cells into the alveolar space and expression of pro-inflammatory molecules in BAL cells of heaves horses.

The observations on the role of PIMs in heaves in horses prompted me to examine the occurrence of PIMs in human lungs. I found a trend for higher numbers of septal macrophages in autopsied lungs from human patients who died of non-pulmonary pathologies compared to those having either COPD or asthma. If these septal macrophages indeed represent the PIMs, this finding is contrary to existing belief that humans, unlike horses, do not have PIMs.

ACKNOWLEDGEMENTS

The following thesis benefited from the insights, direction and support of several people whom I would like to express my gratitude. First, my supervisor, Dr. Baljit Singh, who exemplifies the high quality educator to which I aspire. While veterinary school has the tendency to obliterate any independent thinking, you restored my confidence in the freedom of ideas, challenged them and encouraged them. With your good nature and positive attitude throughout these two years I enjoyed completing this thesis while becoming a wife and a mother.

Next, I wish to thank my advisory committee members: Dr. Hugh Townsend, Dr. Gillian Muir, Dr. Linda Hiebert and especially Dr. Katharina Lohmann for both the valuable insights and technical guidance. Dr. Fernando Marques, and Marianela Lopez for their help with the Bronchoalveolar lavage. The people at the Animal Care Unit for their technical assistance. My lab colleagues and friends: Natasha Thiessen, Dr. Hong Anh Le Minh, Patience Asiamah, David Schneberger and Dr. Sarabjeet Singh, for their help and moral support at late nights and early mornings. It would be failing in my duty if I do not thank Dr. Mariana Amorim, a great veterinarian, researcher and friend, with an endless smile and good mood was always willing to help.

I would like to thank the financial support I received from the Equine Health Research Fund for the project; University of Saskatchewan for the Veterinary Biomedical Sciences Devolved Graduate Scholarship and WCVM Graduate Education Enhancement Fund and Natural Sciences and Engineering Research Council of Canada for my stipend.

To my family, who even from a different continent manage to encourage and support me, my educational pursuits and my dreams. Thank you.

And most importantly I would like to thank my new family. Tal, my husband, who on top of his help with the planning, the execution and the editing of my thesis, is always motivating me and always has a way of seeing the good, I envy you. And to Niv, our little one, for your patience and smile that fills our hearts, thank you.

DEDICATION

To: Morgy, Stormy, Sweety, Lucky, Slim, Lady, Arab, Sam, Flicka and Holly

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ALI:	Acute lung injury
AM:	Alveolar macrophages
ARDS:	Acute/adult respiratory distress syndrome
BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
Cb:	Cubes
COPD:	Chronic Obstructive Pulmonary Disease/Disorder
GC:	Gadolinium chloride
IL-1β:	Interleukin 1 beta
IL-4:	Interleukin 4
IL-5:	Interleukin 5
IL-6:	Interleukin 6
IL-8:	Interleukin 8
INF-γ:	Interferon-gamma
LBP:	Lipopolysaccharide binding protein
LPS:	Lipopolysaccharide
MH:	Mouldy hay
MIP-2α:	Macrophage inflammatory protein-2 alpha
MMP-9:	Matrix metalloproteinase-9
MPO:	Myeloperoxidase
NF-κB:	Nuclear factor-kappaB
PAMP:	Pathogen-associated molecular patterns

PIMs: Pulmonary intravascular macrophages

RAO: Recurrent Airway Obstruction

TCS: Total clinical score

TLR4: Toll-like receptor 4

TLR2: Toll-like receptor 2

TNFα: Tumor necrosis factor alpha

TNCC: Total nucleated cell counts

1. GENERAL INTRODUCTION

1.1. Prevalence and significance in equine industry

Heaves, Broken Wind, and recurrent airway obstruction (RAO) are used to describe a chronic, reversible respiratory tract disease of horses characterized by coughing, mucopurulent discharge from both nostrils, dyspnea and exercise intolerance (McPherson *et al.* 1978; Robinson 2001; Couetil and Ward 2003; Leguillette 2003). Horses older than 7 years of age are most frequently affected, and the prevalence increases with age (Couetil and Ward 2003). In addition, a higher prevalence of heaves affected horses was observed in the Northern Hemisphere, where the horses are housed indoors in relatively humid climate, and hay is not adequately dried (Leguillette 2003). Heaves affects a horse's breathing which impacts mobility. Hence, pleasure horses as well as sport horses are at risk.

1.2. Aetiological factors

Equine heaves resembles those human occupational lung diseases caused by inhaled organic dusts. The dust in the environment of a stabled horse is composed of more than 50 species of moulds, forage mites, endotoxins and inorganic components. The contribution of each of these components to the aetiology of heaves is unknown and perhaps some are synergistic in their pathogenicity (e.g. moulds and bacterial endotoxins). Other, non-specific environmental factors such as cold air and dry air may be important determinants of the day-to-day severity of pulmonary dysfunction (Robinson 2001).

Investigation of the risk factors for airway inflammation in pleasure horses in North America showed that the type of roughage being fed was a major determinant of the number of inflammatory cells in the trachea. Horses eating hay had more neutrophils in the trachea than horses on pasture. Within the hay-fed category, horses eating from round bales had more inflammatory cells than those eating from square bales. Pasture halved the risk for development of heaves compared to eating from square bales (Robinson *et al.* 2006). Hay varies greatly in its dust content, and concentrations of dust in the breathing zone can be over 20 mg/m^3 of air when horses eat from square bales (Woods *et al.* 1993). Horses eating from round bales are likely to have even greater exposure to dust because they keep their faces immersed in the centre of the bale as they eat. Pasture generally provides little dust exposure because of its relatively high water content (Robinson *et al.* 2006). Changing horses' feed from hay to haylage resulted in approximately a 60–70% reduction in mean respirable dust concentration, depending on bedding type. In comparison, changing bedding from straw to wood shavings results in approximately a 23–32% reduction in mean respirable dust concentration depending on feed type (Clements and Pirie 2007).

1.3. Clinical signs

Clinical signs of heaves are caused by the resistance to airflow in the lung due to diffused bronchoconstriction and mucus accumulation. A large number of constricted small bronchi are necessary to impede airflow throughout the lower parts of the lung to the point where clinical signs may appear. The bronchoconstriction is extended to all the lung parenchyma (Leguillette 2003). Exercise intolerance is one of the main complaints

and it usually worsens slowly. The most striking clinical signs are cough, respiratory distress indicated by increased abdominal effort and nasal flaring, mucopurulent nasal discharge, abnormal lung and tracheal sounds and an enlarged lung percussion field (Dixon *et al.* 1995; Robinson 2001). Heaves-affected horses change their breathing pattern and typically have a rapid inspiration and a forced prolonged exhalation. When chronically affected, horses can show hypertrophy of the external abdominal oblique muscles, also called a "heave line" (Leguillette 2003). However, lack of clinical signs or very mild clinical signs, such as occasional cough, or minimal abdominal effort, may render the diagnosis difficult. Rebreathing increases lung sounds, but ancillary diagnostic techniques often are required (Dixon *et al.* 1995; Robinson 2001). Exposure of susceptible horses to moulds may trigger the appearance of clinical signs within 5 h (Brazil *et al.* 2005) to 2 days (Tesarowski *et al.* 1996). When horses are put in a controlled environment or turned out to pasture clinical signs are reversible. Several studies reported an improvement of the respiratory function of heaves horses 1 week after being turned outside on pasture (Leguillette 2003). Others showed remission of signs only after four to six weeks when put in controlled environment or pasture (McGorum *et al.* 1993a; Vandenput *et al.* 1998; Davis and Rush 2002). Subjective clinical scores are used in research and are often based on nasal flaring, abdominal effort, and auscultation (Rush *et al.* 1998b; Courouce-Malblanc *et al.* 2007).

1.4. Bronchoalveolar lavage (BAL)

In healthy horses, the major cell populations in the BAL fluid (BALF) consist of alveolar macrophages (AM) (60%) and lymphocytes (35%). The other cell types –

neutrophils, eosinophils, mast cells and epithelial cells – are usually in negligible numbers (Robinson 2001). The most consistent cytologic abnormality in BALF from horses with heaves is neutrophilic inflammation with neutrophils forming 50-70% of the total cell count (Rush *et al.* 1998a; Robinson 2001). Comparison of cellular and molecular components of BALF harvested from different segments of the equine lung suggest that a single BALF sample is representative of the entire lung in these horses (McGorum *et al.* 1993b).

BAL analysis is one of the most consistent tools used to diagnose horses with lower respiratory pathology. However, there are inconsistencies in the reports regarding the order of appearance of the changes in BAL findings compared to the appearance of changes in the clinical signs. This point will be discussed in detail in section 1.6.

1.5. The type of immune reaction, cells and cytokines involved in the pathogenesis of heaves

In 1971 the term 'equine chronic obstructive pulmonary disorder (COPD)' was introduced by Sasse to describe the syndrome of airway inflammation and obstruction that affects the mature horse (Sasse 1971). This name portrays the chronic nature of the disease and the obstruction of the airways associated with it. In addition, the mucus and neutrophilic accumulation in the airways greatly resemble human lung disease of the same name. With the growing understanding of the pathogenesis of heaves in horses, the term COPD is now thought to be misleading; human COPD is a progressive disease with little reversibility which usually results from smoking. However, heaves is characterised by reversible airway narrowing due to bronchospasm that more closely resembles human

asthma (Robinson 2001). The type of immune reaction, including the cells and cytokines involved in equine heaves, should also be taken into account when choosing an accurate terminology for this disease.

Neutrophilic pulmonary inflammation is the most consistent BALF abnormality in horses with heaves during disease exacerbation. There are contradictory results in macrophage numbers and total cells counts in horses with heaves which may be due to differences in disease duration, environmental antigen exposure, standardization of BAL technique, secondary bacterial infection and disease severity in the study population (Rush *et al.* 1998a).

Clinical signs of heaves are exacerbated following exposure of the horses to dusty hay which suggests an allergic reaction to the moulds and fungi in the hay (Lavoie *et al.* 2001). In addition, the positive passive cutaneous anaphylaxis test observed using sera from horses with heaves (Eyre 1972) as well as the elevated levels of IgE in BAL of affected horses (Schmallenbach *et al.* 1998; Horohov *et al.* 2005) points to a *type I* hypersensitivity reaction. On the other hand, the neutrophilic inflammation in airways of horses with heaves could suggest an involvement of a *type III* hypersensitivity reaction (Lawson *et al.* 1979; Madelin *et al.* 1991). Inconsistencies were also found when attempting to define the predominant T-cell population, CD4 or CD8 T-cells, in blood and BAL of horses with heaves (McGorum *et al.* 1993b; Watson *et al.* 1997; Kleiber *et al.* 1999). The further divergence in CD4+ T helper cells (Th) into Th1 and Th2 subsets was extensively studied for better understanding of the pathogenesis of chronic airway inflammatory diseases. Cytokines such as IL-4, IL-5 and IL-13 that are produced by Th2 cells are associated with allergic inflammation, while interferon-gamma (INF-γ) is

associated with cell-mediated immunity which characterizes the Th1 response. BAL cells retrieved from heaves affected horses were shown to have a mixed response and while some reports showed an increased expression of IL-4 and IL-5 mRNA, (Cordeau *et al.* 2004) others showed an increased expression of INF- γ (Giguere *et al.* 2002). Taking together all of the above, it is suggestive that heaves is a combined response with both an allergic component as well as an inflammatory one. The variation between studies might be a result of methodological factors, disease definition and sampling time (Robinson 2001; Horohov *et al.* 2005).

Neutrophils are widely known as the host defence against bacterial pathogens as these cells release toxic proteases and oxygen-free radicals. However, neutrophils were also shown to play a major effector role in lung diseases that are of non-infectious origin, including acute asthma in humans (Ordonez *et al.* 2000) and acute respiratory distress syndrome (Nys *et al.* 2002). Occupational pulmonary diseases, such as cotton worker's lung, are another group of human non-infectious pulmonary disorders that is mediated by neutrophils and to a great extent parallels equine heaves (Liebers *et al.* 2006). The morphology of neutrophils isolated from BALs of heaves-affected horses differs from the cytological findings in pulmonary bacterial infections in that the neutrophils appear normal, without evidence of toxicity or bacterial phagocytosis. In addition, the epidemiological pattern of heaves, characterized by individual older horses being affected, contrasts with the pattern in infectious respiratory disease, where large numbers of in-contact horses (especially younger animals) are affected. The widely accepted viewpoint that neutrophils equal infection and eosinophils equal allergy has recently been challenged. While the eosinophils are indeed the major inflammatory cells in the airways

of chronic stable asthmatics, the neutrophils have recently been implicated as the predominant cells in acute severe asthma in human patients (Ordonez *et al.* 2000; Robinson 2001).

1.6. The role of neutrophils in equine heaves

Although investigators have correlated the proportion of neutrophils in airway secretions with clinical disease severity (Vrins *et al.* 1991) and neutrophils are consistently recruited to the airspaces of susceptible animals after at least 5 h of challenge (Brazil *et al.* 2005), some studies have demonstrated accumulation of neutrophils without evidence of airway obstruction (Freeman *et al.* 1993; Tremblay *et al.* 1993) and in some animals airway dysfunction develops prior to or without significant pulmonary neutrophil sequestration (Naylor *et al.* 1992). These discrepancies may reflect the heterogeneity in the inflammatory and functional airway responses within a population of animals classified as heaves-susceptible as well as the low sensitivity of conventional pulmonary function tests. Nevertheless, it is still well believed that recruitment of neutrophils to lungs of heaves susceptible horses is a major component in the induction and maintenance of the disease (Brazil 2001). In heaves-susceptible horses neutrophil recruitment was shown to be induced by hay and straw challenge (Jackson *et al.* 2000) and by inhalation of endotoxin (Pirie *et al.* 2001) and aqueous mould extracts (Pirie *et al.* 2002). These challenge agents stimulate production of an array of chemotactic mediators such as IL-8 and macrophage inflammatory protein-2 alpha (MIP-2α) by resident airway cells (e.g. alveolar macrophages and epithelial cells) (Franchini *et al.* 1998). Following short duration (5 h) challenge neutrophil numbers in BALF may rise 50-fold, reaching a

peak approximately 24 h after challenge began. Neutrophil numbers then fall, returning to baseline levels by 4 days (Brazil *et al.* 2005). Neutrophil kinetics during prolonged challenge are less well characterised. Persistence of increased nuclear factor-kappaB (NF-κB) activity in bronchial cells and accompanying lung dysfunction has been reported up to 21 days after removal of horses from the challenge environment (Bureau *et al.* 2000a; Bureau *et al.* 2000b).

There is substantial evidence for neutrophil activation *in vivo* and lung injury in heaves. One such evidence is the direct measurement of neutrophil secretory products (usually proteinases) in BALF; increased levels of matrix metalloproteinase-9 (MMP-9) were detected in BALF of heaves-susceptible horses after inhalation of different dust components (Raulo *et al.* 2001; Simonen-Jokinen *et al.* 2005). The magnitude of airway neutrophilia also closely correlated with marked increase in BALF levels of equine neutrophil elastase (ENE) 2A (Deaton *et al.* 2005). Myeloperoxidase (MPO) concentration, a marker of neutrophil accumulation and activation as well as the absolute and relative neutrophil counts, was shown to be greater in BALF of horses with an RAO crisis than in the control horses. In contrast, blood neutrophil counts and plasma MPO values were similar in both groups (Art *et al.* 2006).

Additional evidence is the detection of neutrophil-mediated disruption of pulmonary homeostasis. Acute heaves is associated with a significant increase in markers of oxidative stress. The glutathione status in the pulmonary epithelium lining fluid was found to be significantly different in heaves-affected horses in acute crisis compared to healthy horses, indicating the occurrence of oxidative stress. When heaves-affected horses were in crisis their reduced glutathione and total glutathione remained unchanged

but their oxidised glutathione and glutathione redox ratios were significantly increased compared to remission, which supports the hypothesis that oxidative stress is associated with lower airway disorders occurring in heaves (Art *et al.* 1999).

In addition to the cytotoxic granules released by activated neutrophils, these primed neutrophils are also able to generate and release soluble mediators, with significant inflammatory regulatory role. Hence, neutrophils may become the dominant source of pro-inflammatory cytokines which drive the ongoing airway inflammation (e.g. TNFα, IL-8, IL-1β, IL-6 and MIP-2) (Brazil 2001).

Increased numbers of apoptotic neutrophils have been observed in BALF during the resolution phase of an acute episode of heaves and more particularly an increase in alveolar macrophages that have ingested intact apoptotic neutrophils was detected during this stage (Brazil *et al.* 2005). Nevertheless, during crisis, granulocytes retrieved from BALF of heaves susceptible horses demonstrated a significant delay in apoptosis compared with blood granulocytes from the same horses and blood and BALF granulocytes from healthy horses (Turlej *et al.* 2001).

1.7. The contribution of endotoxin to the pathogenesis of Heaves

Inhaled endotoxins are an important cause of human pulmonary disease, with the severity of pulmonary inflammation and clinical symptoms experienced by subjects exposed to organic dusts being related to the endotoxin concentration of the inhaled dust (Smid *et al.* 1994; Vogelzang *et al.* 1998). In addition, the severity of human asthma has been related to the level of endotoxin exposure (Michel *et al.* 1991; Rizzo *et al.* 1997), suggesting that inhaled endotoxin may potentiate the inflammatory response to allergens

in atopic subjects. Several studies were performed in an attempt to understand the contribution of endotoxins in the pathogenesis of heaves. Pirie et al. suspected that the method of mould extract production is likely to result in endotoxin contamination, and therefore measured the response of heaves-susceptible horses to *Aspergillus fumigatus* extract inhalation before and after LPS depletion. Depletion of LPS from the mould extracts resulted in a significant reduction in airway neutrophil numbers and increase in arterial oxygen tension (Pirie *et al.* 2003b). The inflammatory response was shown to be re-established by adding endotoxin back to the endotoxin-depleted hay dust (Pirie *et al.* 2003a). An earlier investigation, conducted by the same group, on the pulmonary effects of inhaled endotoxin in control and heaves-susceptible horses showed a no-response threshold lower for the heaves group (<20 μg for airway inflammation; 200 to 2000 μg for lung dysfunction) than for the control group (20 to 200 μg for airway inflammation; >2000 μg for lung dysfunction). Comparison of the effects of acute LPS inhalation and hay/straw challenges suggested that inhaled endotoxin is not the sole cause of heaves. However, a 5 h exposure to poor air hygiene equates to an LPS dose of 160 μg. This dose exceeds the threshold dose of LPS which causes inflammation in horses with asymptomatic heaves (20 μg). Hence, endotoxin is likely to contribute to airway inflammation, both in heaves horses and in normal horses when they are exposed to high levels in poor stable environments (Pirie *et al.* 2001).

1.8. Toll-like receptors

As mentioned above, microbial-derived products, such as endotoxin, were shown to play an important role in the pathogenesis of heaves. Pathogen-associated molecules or

Pathogen-Associated Molecular Patterns (PAMP) enable innate immune cells to discriminate between 'self' and 'non-self' since they are specifically generated by the pathogen and not by the host. These PAMPs are recognized by pattern recognition receptors such as the Toll-like receptors (TLRs). The prototypic receptor Toll was first identified in the fruit fly *Drosophila*, but there are at least 13 identified mammalian TLRs, to date, that share similarities in their extracellular and intracellular domains (Brikos and O'Neill 2008). The TLRs act as primary sensors that detect a wide variety of microbial components and elicit innate immune responses. All TLR signalling pathways culminate in activation of the transcription factor NF-κB, which controls the expression of an array of inflammatory cytokine genes (Uematsu and Akira 2006; Kawai and Akira 2007). TLR2 and TLR4 are the best characterized TLRs. TLR4 is crucial for the recognition of endotoxin, in particular lipopolysaccharide (Schwartz 2001; Togbe *et al.* 2007), and this is supported by the fact that TLR4-deficient mice were shown to be hyporesponsive to LPS (Hoshino *et al.* 1999). TLR4 is expressed in a variety of cell types within the lung, including pulmonary epithelial cells (Monick *et al.* 2003; Ainsworth *et al.* 2006), alveolar macrophages (Fernandez *et al.* 2004; Singh Suri *et al.* 2006), endothelial cells (Andonegui *et al.* 2003; Singh Suri *et al.* 2006) and airway smooth muscle cells (Morris *et al.* 2005), and its expression can be stimulated by LPS itself (Singh Suri *et al.* 2006). Stimulation of TLR4 leads to production of cytokines, such as interleukin-8 (IL-8) (Gon *et al.* 2004), TNFα, and IL-6 (Gon *et al.* 2004; Jeyaseelan *et al.* 2005; Baumgarten *et al.* 2006). TLR2 recognizes various microbial components, such as lipoproteins/lipopeptides and peptidoglycans from gram-positive and gram-negative bacteria, and lipoteichoic acid from gram-positive bacteria, a phenol-soluble modulin

from *Staphylococcus aureus*, and glycolipids from *Treponema maltophilum* (Takeuchi *et al.* 1999; Akira *et al.* 2006).

The TLRs were shown to be crucial not only in mediating innate immunity, but specifically in the development of acute lung inflammation. Jeyaseelan et al. demonstrated that LPS-induced acute lung injury (ALI) in a mouse model is partially dependent on CD14 but fully dependent on TLR4, and that blocking TLR4 reduced the characteristic features of ALI, including an increase in the total WBC count, neutrophil influx and sequestration in the lung (Jeyaseelan *et al.* 2005). In addition, by use of TLR4-deficient mice it was demonstrated that TLR4-dependent signalling cascades are critical for the induction of inflammatory cytokines (TNF, IL-1β, IL-6) and NO production after LPS exposure in the lung (Baumgarten *et al.* 2006). Recent investigations on the nature of TLRs in the horse's lung revealed expression of TLR4 but not TLR2 in normal horse lungs; however an increase in lung expression of both TLR4 and TLR2 mRNA was observed 2 h after intravenous administration of LPS (Singh Suri *et al.* 2006). In addition, Berndt et al. demonstrated an involvement of TLR4 in the pathogenesis of heaves by showing an increase in TLR4 mRNA expression in bronchial epithelial cells from heaves horses exposed to stable dust and this increase in TLR4 mRNA correlated with IL-8 mRNA expression as well as with neutrophilic airway inflammation (Berndt *et al.* 2007). Similar to heaves, environmental exposure to endotoxin has a crucial role in immune maturation and development of asthma, therefore TLR4 was suggested to be involved in this pathogenesis. Indeed, polymorphisms in the TLR4 gene were associated with a modified response to endotoxin in asthmatic patients (Werner et al. 2003; Adjers et al. 2005).

1.9. Pulmonary Intravascular Macrophages (PIMs)

In the lung, as in several other organs in the body, macrophages reside in various anatomical compartments. For example, in addition to well characterized alveolar macrophages, the lung also contains interstitial, airway and, in some species, intravascular macrophages. Mononuclear phagocytes constitute a dynamic population of cells in the lung. Large numbers of monocytes pass through the pulmonary circulation and may become transiently sequestered in pulmonary capillaries. Some of these sequestered cells migrate into the interstitium or alveolar spaces, and differentiate into mature macrophages. In some domestic animal species such as cattle, goats, sheep and pigs (Atwal and Saldanha 1985; Warner *et al.* 1986; Wheeldon and Hansen-Flaschen 1986; Winkler 1988; Singh *et al.* 1995; Brain *et al.* 1999), monocytes permanently adhere to the endothelium and differentiate into mature pulmonary intravascular macrophages (PIMs). One of the earliest reports showing the presence of PIMs in calf lungs was published in 1974 (Rybicka *et al.* 1974), followed by the evidence of erythrophagocytosis by PIMs in goats (Atwal and Minhas 1992). The PIMs in the equine lungs were first described in 1992 by Atwal and colleagues (Atwal *et al.* 1992) and subsequently in 1994 by Longworth and colleagues (Longworth *et al.* 1994). There are reports of occurrence of smaller populations of PIMs in rabbit and cat lungs as well (Brain *et al.* 1999). In pigs and sheep, the PIMs were shown to be sequestered within 4 weeks of their birth (Winkler and Cheville 1985, 1987). However, there are no data on the ontogeny of PIMs in horses. Currently, there are no molecular or evolutionary explanations for the intriguing recruitment of PIMs in some host animal species and their absence in others, such as dogs and rats.

It has been known for many years that PIM-containing animal species are more prone to lung inflammation (Frevert and Warner 1992). For example, an injection of 0.003-1.3 μg of *E. coli* LPS/kg body weight induces pulmonary hypertension and cardio-pulmonary shock in horses (Frevert and Warner 1992). In addition when *Pseudomonas aeruginosa* bacteria are intravenously injected into sheep or pigs, the pulmonary capillaries become congested with red cells, neutrophils, platelets, lymphocytes and fibrin clumps, and intravascular macrophages. These morphologic changes accompany physiological changes, such as pulmonary hypertension and hypoxemia. In contrast, rats injected intravenously with *Pseudomonas aeruginosa* show almost no pulmonary morphologic changes and no features of pulmonary failure. In the rat, most bacteria are retained in the liver by Kupffer cells, and it is the hepatic sinusoids that are congested with neutrophils and platelets (Crocker *et al.* 1981; Warner *et al.* 1987). A series of experiments provided conclusive linkage between PIMs and pulmonary responses in sheep. Neonatal lambs have very few PIMs and show no hemodynamic response to Monastral blue or liposomes (Longworth *et al.* 1992). However, PIM sequestration in lambs by two weeks of age is accompanied by enhanced vascular response to the same treatment; this hypertensive response is eliminated by removing the PIMs (Sone *et al.* 1997).

Interestingly, depletion or inactivation of PIMs in adult horses also blocked endotoxin-induced increase in pulmonary arterial pressures and reduced expression of IL-1β (Longworth *et al.* 1996; Parbhakar *et al.* 2005). Endotoxin-induced pulmonary vascular responses are generally attributed to production of vasoactive substances such as thromboxanes by the PIMs. This contention is supported by *in vitro* data showing that

porcine PIMs produce more arachidonic acid metabolites compared to the alveolar macrophages (Chitko-McKown *et al.* 1991; Chitko-McKown and Blecha 1992). Taken together, the evidence shows a causal relationship between PIMs and endotoxin-induced pulmonary vascular responses.

The uptake of endotoxins and other bacterial products is done through molecules such as Toll-like receptors and CD14 (Aderem 2001). Recently, the first data on the expression of TLR4 in PIMs in horse (Singh Suri *et al.* 2006), cattle and pigs (Wassef *et al.* 2004; Singh Suri *et al.* 2006) was reported. TLR4 expressed in PIMs seems to be a major component of total TLR4 expression in horse lungs since depletion of these cells significantly reduced lung expression of TLR4. Although TLR2 protein expression was not examined, TLR2 mRNA expression was not detected in lungs from normal horses. The strategic location of TLR4-enriched PIMs provides them with unique opportunities to sense and respond to blood-borne and perhaps also air-borne endotoxins. Indeed, TLR4 was shown to colocalize with *E. coli* LPS in cytoplasm and nucleus of equine PIMs (Singh Suri *et al.* 2006). The uptake of LPS by equine PIMs is in line with their previously reported significant phagocytic activity for tracer particles (Singh *et al.* 1994; Singh *et al.* 1995).

PIMs location in lung microvessels creates a unique structural potential for exaggerated pulmonary response to endotoxins. It is known that interaction of endotoxin with TLR4 or the uptake of LPS by macrophages activates them (Fujihara *et al.* 2003). Macrophages, once activated, secrete mediators such as IL-1β and TNFα to induce expression of adhesion molecules on lung microvascular endothelium (Springer 1994; Antonelli *et al.* 2001). Furthermore, production of metabolites such as thomboxanes in

lung microvessels modifies pulmonary hemodynamics. As a result, neutrophils start to accumulate in the lungs and amplify lung inflammation through free oxygen radicals and cytokines. Based on the role of macrophages in endotoxin-induced responses in general, it is conceivable that TLR4 expressing PIMs will amplify lung inflammation in horses. This contention seems to be true based on data from PIM-depletion studies in sheep, cattle and horses (Sone *et al.* 1997; Sone *et al.* 1999; Singh *et al.* 2004; Parbhakar *et al.* 2005).

There is still a great gap in our knowledge of the biology of the PIMs due to our inability to isolate them. PIMs are intimately attached to the capillaries' endothelium and hence cannot be removed even with drastic enzyme treatments (Rogers *et al.* 1994). Our growing understanding of the pathophysiological role of PIMs is through *in vivo* studies of selectively depleting or inactivating the PIMs and comparing the outcomes between non-depleted and depleted animals. Gadolinium chloride, a rare earth lanthanide, was shown to kill and eliminate macrophages as well as interfere with their phagocytic function (Mizgerd *et al.* 1996). In species having PIMs, the macrophages affected the most by intravenous GC injection are the PIMs due to the "first pass" of the chemical through the lung following administration in jugular vein (DeCamp *et al.* 1992; Staub 1994; Singh and de la Concha-Bermejillo 1998). Consequently there may be little spill over of GC to Kupffer cells thus reducing the possibility of damage to these cells. Gadolinium chloride has been used to remove PIMs in several animal species such as sheep, calves and horses (Singh and de la Concha-Bermejillo 1998; Singh *et al.* 2004; Parbhakar *et al.* 2004).

The role of PIMs in the development of RAO in horses has not been addressed. One major difference between this pathology and those discussed above regarding the PIMs is that the dust and endotoxin in RAO are introduced via the airway and are not blood borne. There are some studies that demonstrate the involvement of PIMs in lung infections. For example, after intratracheal inoculation with *Haemophilus pleuropneumoniae*, the relative volume of intravascular macrophages in pig lungs increased in areas of inflammation and necrosis and it was concluded in this study that the intravascular macrophages clear cellular and acellular debris from the blood in pneumonitis (Bertram 1986). A similar role has been proposed for PIMs in the pulmonary response to intratracheal inoculation of *Mannheimia haemolytica* (Whiteley *et al.* 1991; Singh *et al.* 2004) in calves and in experimentally induced African Swine Fever in pigs (Sierra *et al.* 1990; Carrasco *et al.* 1992; Carrasco *et al.* 2002).

Much of the current research focuses on whether PIMs are induced in humans in certain pathological conditions. Human lungs do not contain PIMs under normal circumstances and foreign particles in the circulation are localized in the liver, phagocytised by Kupffer cells (Warner 1996; Longworth 1997). However, gram-negative septicaemia or endotoxemia in humans often leads to acute lung injury or Adult Respiratory Distress Syndrome (ARDS) and it is not understood how pulmonary inflammation develops from systemically introduced pathogens. Sheep and pigs are both used as experimental models for ARDS in humans (Eiznhamer *et al.* 2004; Wang *et al.* 2008). Because PIMs are believed to be absent in humans, the use of sheep and pigs as a model to study human lung inflammation may not provide applicable data. There is, however, a possibility of recruitment of PIMs in the lungs of humans following

physiological stress which makes their pulmonary vasculature similar to that in species with resident intravascular macrophages (Longworth 1997). One ultrastructural report showed PIMs in biopsies taken from human patients undergoing thoracotomies for excision in noninfectious lung diseases (Dehring and Wismar 1989). In addition, there is some indirect evidence to support accumulation of mononuclear phagocytes in lung vasculature of patients with liver disease (Keyes *et al.* 1973; Imarisio 1975). As mentioned above, the pathogenesis of RAO in horses has some similarities mainly to human asthma, but also to COPD and human occupational lung diseases, and exposure to the triggering molecules in all these pathologies occurs though the airways. Hence, studying the role of PIMs in the development of heaves in horses may shed some light on the role of these cells in several human pulmonary pathologies.

2. HYPOTHESIS

My working hypothesis is that the depletion of PIMs in horses will attenuate the activation of airway inflammatory cells and reduce proinflammatory cytokines production in the development of heaves and in consequence will attenuate clinical signs of heaves and lower the response of BAL cells to a secondary challenge with LPS.

3. OBJECTIVES

The study was undertaken to investigate:

3.1. the effect of PIM depletion in horses on:

3.1.1. the development of clinical signs of heaves

3.1.2. the total alveolar cells, alveolar macrophages and neutrophils in the alveolar space in the development of heaves

3.1.3. the activation state of BAL cells

3.1.4. the activation state of BAL cells after a secondary *in vitro* challenge with LPS

3.2. the presence of septal macrophages in human lungs from healthy and COPD or asthmatic patients

4. MATERIALS AND METHODS

4.1. Animals

Nine light type mares (400-600 kg), 10 to 20 years of age, with a history of recurrent airway obstruction in the last two years were studied. The mares were kept in outdoor paddocks with sheds. They were maintained on alfalfa cubes and challenged with mouldy hay and had free access to water and trace-mineralized salt, in accordance with the University of Saskatchewan's Animal Care and Use Committee. Mares had no signs of an ongoing systemic disease at initial physical examination. Episodes of heaves were induced by feeding the horses with mouldy hay (2 year old hay) in a round bale feeder for 7 days and remission was achieved by feeding the horses with alfalfa cubes for a period of 3 weeks (Figure 4.1).

Physical examination including heart rate, respiratory rate, rectal temperature, auscultation with and without a rebreathing bag and evaluation of nasal flaring, nasal discharge, and abdominal effort was performed periodically, before and after treatments and prior to all BALs. A subjective clinical assessment of the respiratory effort was done using a Total Clinical Score (TCS) system adapted and modified from previous published studies (Naylor *et al.* 1992; Rush *et al.* 1998a; Berndt *et al.* 2007; Courouce-Malblanc *et al.* 2007). The intensities of the clinical signs were scored from normal to severe as indicated in Table 4.1. Results represent one time point of clinical assessment done for each treatment just prior to the BAL procedure. The TCS was calculated for each mare by adding the values for each clinical sign; hence the TCS can range between 4 to 13. In addition the effect of the treatments on each clinical sign was evaluated by calculating the sum of scores from all mares for each clinical sign.

Table 4.1. Total clinical scores parameters and their description

Parameter/Clinical sign	Score	Description
Abdominal effort	1	Normal (no signs of dysfunction)
	2	Moderate abdominal component
	3	Severe, marked abdominal component
Nasal flaring	1	Normal (no signs of flaring)
	2	Moderate nostril flaring
	3	Severe, continuous flaring during each respiration
Coughing	0	No coughing
	1	Coughing during the examination
Auscultation	1	Normal lung sounds
	2	Occasional wheezes and crackles
	3	Wheezes and crackles during each respiration
Auscultation after a rebreathing bag	1	Normal lung sounds
	2	Occasional wheezes and crackles
	3	Wheezes and crackles during each respiration

Figure 4.1. Photos of mares in the study demonstrating the different eating patterns when fed with: hay in round bales (a,b) and alfalfa cubes (c)

4.2. Study design

Horses were randomly assigned to two treatment groups with five mares in one group and the remaining four in the second group. The study design was a modified cross-over, in which all horses ultimately received all treatments. The treatments included: **1**. pasture/cubes no gadolinium chloride (GC) treatment **(Cb)** – remission or non-heaves induced horses; **2.** Mouldy hay no GC treatment **(MH)** – heaves induced horses; **3.** Pasture/cubes and intravenous administration of GC **(Cb-GC); 4.** Mouldy hay and intravenous administration of GC **(MH-GC)**. The order of the treatments was different in each group. Horses in group 1 (n=5) received treatment 1 to 4 in the order stated above, while horses in group 2 (n=4) received treatment in the order 3, 4, 1 and 2. Following induction of heaves, horses were fed with alfalfa cubes for 3 weeks to achieve remission. In addition, after performing BALs horses were fed with cubes for 2 weeks to allow reconstitution of the alveolar cells as well as recovery from the irritation that occurred during the BAL procedure. For the mouldy hay challenges, horses received 3 treatments of GC, the first 2 days prior to the MH while the horses were still on cubes, with 2 more repeats at 96 h intervals. The third administration occurred one day prior to the BAL. The treatment plan is summarized in Figure 4.2.

Whole blood was collected in EDTA tubes prior to each BAL for complete blood cells counts and differentials

.

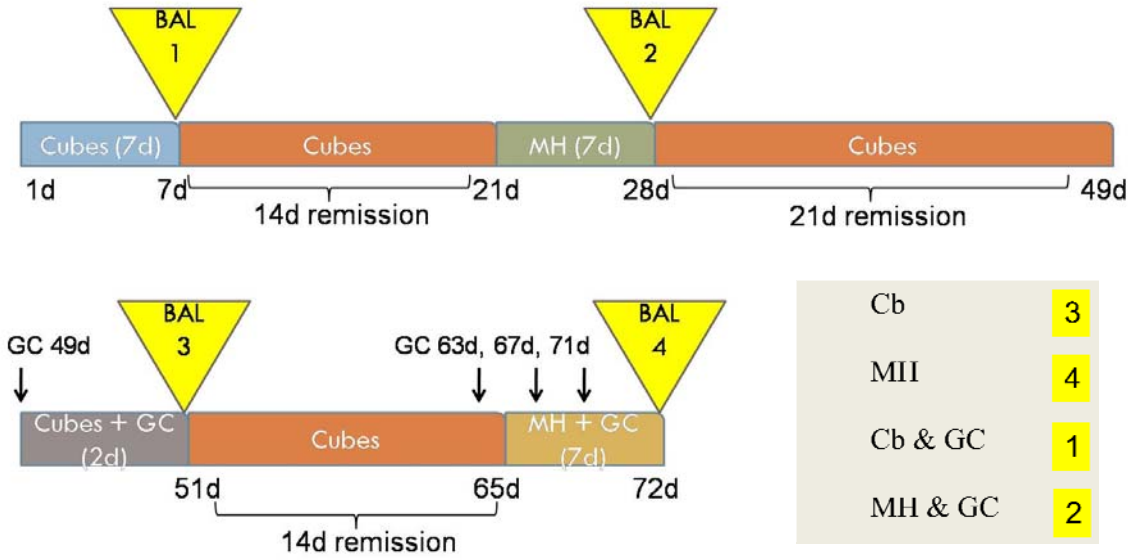

Figure 4.2. A schematic summary of the treatment plan. The box on the right indicates the order for group 2.

4.3. Gadolinium chloride treatment

Gadolinium Chloride, GdCl3 · 6H2O (Sigma-Aldrich, Oakville, ON, Canada), was administered at a dose of 10 mg/kg intravenously (jugular vein) in 500 ml of saline over 30 min. Heart rates were recorded before and after the infusion. It has been previously shown that this dose of GC causes significant depletion of PIMs and new PIMs are recruited after 72 h of the treatment (Parbhakar *et al.* 2004). The GC treatments are outlined in the preceding section.

4.4. Bronchoalveolar lavage

Horses were sedated with acepromazine (0.02 mg/kg intravenous) followed by detomidine (0.01 mg/kg intravenous) and butorphenol (0.01 mg/kg intravenous). A 3m

video-endoscope (Olympus SIF-100, Olympus Canada Inc., Markham, ON, Canada) was passed intranasally and directed to the right cranial lobar bronchus to instill 500 ml of warm solution of sodium chloride 0.9%, sodium bicarbonate 0.06%, pH 6.5, at 37^0C. Following instillation, the fluid was aspirated with a pump (Millipore GE 5KH33DN16X mechanical pump, Mississauga, ON, Canada) and collected and stored in sterile flasks on ice for further analysis. The pressure of the pump was adjusted to prevent airway collapse. The procedure was repeated for the left lung. On each side of the lung about 20 ml of 2% Lidocaine HCl (Ayerst Veterinary Labs, Guelph, ON, Canada) was injected prior to lavage to relieve the irritation and minimize the coughing. Total volumes of BALF were recorded.

4.5. BALF processing

After recording the total volumes, the BAL fluids were centrifuged at 2000rpm for 10min. Two hundred millilitres of the supernatant were collected from each BALF, aliquoted and stored at -80^0C. The cells were washed with RPMI-1640 medium (Invitrogen, Burlington, ON, Canada) containing 2 mM L-glutamine, 10mM Hepes, and 5μg/ml gentamicin (Invitrogen, Burlington, ON, Canada) and were centrifuged again at 400 g for 10 min. The supernatant was discarded and the cells were resuspended in RPMI-1640 medium containing 2 mM L-glutamine, 10 mM Hepes, 5 μg/ml gentamicin, and 10% horse serum (Invitrogen).

4.6. Analysis of cell populations in the BALF

Total nucleated cell counts (TNCC) were determined by use of a hemocytometer. Total nucleated cell concentration (number of cells/ml) was calculated by dividing the TNCC by the corresponding volume of the BALF. The differential cell counts were determined by examination of 100 consecutive leukocytes on a cytospin preparation stained with Giemsa stain. Neutrophils and alveolar macrophages concentrations were calculated by multiplying the percentage of cells with the total nucleated cells concentrations. The evaluator performing the cell counts was blinded to the treatments.

4.7. *In vitro* LPS challenge

Cells were distributed to Eppendorf tubes at a volume of 1ml in each tube, and a concentration of $5X10^6$ cells/ml. The cells were stimulated with *Escherichia coli* LPS (1 μg/ml, strain 0127:B8) (Sigma-Aldrich, Oakville, ON, Canada) or media (control) for different time points (0, 5, 10, 15, 30, 60 min and 6 h). Following each time point the cells were spun down. The supernatants were collected and stored at -80^0C and cells were frozen in liquid nitrogen and stored at -80^0C.

4.8. Whole blood processing

Forty millilitres of whole blood was collected in heparin vacutainer tubes. Sedimentation of red blood cells was done by mixing the whole blood with a 6.5% T75 solution of Dextran (1:1 v/v) and allowing rouleaux formation over a one hour period. The leukocyte/platelet-rich plasma layer above was then aspirated and centrifuged at

400 g for 10 min. The remaining red blood cells were lysed by adding 9 ml of hypotonic solution (distilled water) to the cell pellet for 18sec following by 1 ml of 10X concentrate HBSS (Sigma-Aldrich, Oakville, ON, Canada) to stop the lysis. The cells were centrifuged again at 400 g for 10 min, counted using a hemocytometer and were resuspended with RPMI-1640 medium containing 2 mM L-glutamine, 10 mM Hepes, 5 μg/ml gentamicin, and 10% horse serum (Invitrogen) to a final concentration of $5X10^6$ cells/ml. The cells were distributed to two Eppendorf tubes (1 ml/each), spun down and after discarding the supernatant the pellets were frozen in liquid nitrogen and stored at -80^0C.

4.9. Enzyme linked- immunosorbent assay (ELISA) for tumor necrosis factor-α (TNFα)

TNFα protein concentrations were determined using the equine TNFα ELISA screening set (Pierce, Endogen, Rockford IL, USA) both in the BAL supernatants that were obtained immediately following BAL collection (after the first centrifugation, representing the 0 h time point), and in cell supernatants that were collected from the BAL cells after 6 h of incubation with or without LPS. The sandwich ELISA method was performed according to the manufacturer's instructions. Samples from the 9 mares were loaded in duplicate wells (100 μl in each well). Negative controls (media, media + LPS (1 μg/ml) and reagent diluent) were also loaded in duplicate wells. Following addition of the stop solution, absorbance was measured using BMG-Labtech's-NOVOstar-Microplate-Fluorometer (Biocompare, South San Francisco, CA, USA) at a 450 nm wavelength.

4.10. RNA isolation and cDNA synthesis

Total RNA was extracted from BAL cells and whole blood cells using an RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. The integrity of RNA as well as its quantification was evaluated using a Thermo Scientific NanoDrop spectrophotometer (Thermo Fisher Scientific, ON, Canada). Following DNase treatment, the mRNA was reverse transcribed at 42ºC for 30 min by using QuantiTect Reverse Transcription kit (Qiagen, Mississauga, ON, Canada) as per manufacturer's instructions.

4.11. Quantitative real time reverse-transcriptase polymerase chain reaction (qRT-PCR)

The cDNA from the BAL cells was used for qRT-PCR analysis for the expression of tumor necrosis factor alpha (TNF alpha; GenBank Accession No. EU438779), interleukin 8 (IL-8; GenBank Accession No. AF062377), and toll-like receptor 4 (TLR4; GenBank Accession No. AY005808) genes using QuantiFast SYBR Green PCR kit (Qiagen, Mississauga, ON, Canada), as per manufacturer's instructions. The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH; GenBank Accession No. AF157626) was used as the reference housekeeping gene. The reactions were performed using the primer pairs; 5'-TGTAGCAAACCCCCAAGCCGA-3' and 3'-GCGGCTGA TGGTGTGGGTGAG-5' for TNFα; 5'-ATGACTTCCAAGCTGGCTGTT-3' and 3'-ATGATTTCTGAGTTTTCGCAG-5' for IL-8; 5'-GCCTTCACTACAGAGAC TTCATTC-3' and 3'-GGGACACCACGACAATAACTTTC-5' for TLR4; and 5'-TCACCATCTTCCAGGAG-3' and 3'-GTCTTCTGGGTGGCAG-5' for GAPDH. Real-

time PCR analysis was performed using the MX3005P LightCycler (Stratagene, La Jolla, CA, USA). The cDNA was denatured at 95°C for 5 minutes. This was followed by amplification of the target DNA through 45 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 60°C for 45 seconds. Relative expression levels (fold differences) were calculated after correction for expression of GAPDH, and relative to the results in the Cb treatment, using MxPro software (Stratagene).

4.12. Collection of total airborne dust

Total dust samples were collected from each setting environment (cubes and mouldy hay) as previously described (Kirychuk *et al.* 1998; Charavaryamath *et al.* 2005). Briefly, an air sampling pump (SKC Aircheck Model 224-PCXR4, SKC Inc., Eighty Four, PA, USA) was connected to sampling cassettes (SKC Inc.) placed in cassette holders (SKC Inc.). A 37 mm Polyvinyl Chloride (PVC) filter, 5.0 µm pore size (SKC Inc.) was placed in each cassette. While collecting the dust, the pump was placed in a bag which was carried by an operator and the cassettes were held 20-40 cm from the nostrils of a randomly chosen horse to collect airborne dust present in a horse's breathing zone. This provides a more accurate indication of the amount of dust entering the respiratory tract than remote sampling. The pump flow rate (2.1 liter/min) was calibrated before and at the end of each sampling period using an air flow calibrator (Bios Dry-Cal DC-Lite Calibrator, Bios International Corporation, Butler, NJ, USA). The sampling period for both environments was 1h, and the same horse was used for both environments. Gravimetric analysis for total dust (milligram of dust/m^3 of air [mg/m^3]) was performed

by weighing the filters 3 times before and after dust collection and averaging the results. The method for calculating the total dust concentration was performed according to the NIOSH Manual of Analytical Methods (NMAM), *fourth edition* (Clere and Heart 1994). The filters were then placed in 50 ml polypropylene centrifuge tubes and stored at 4°C until endotoxin analysis was performed.

4.13. Endotoxin evaluation

Ten millilitres of sterile, nonpyrogenic water (LAL Reagent Water; BioWhittaker, Walkersville, MD) were added to each tube containing the filters and the Chromogenic-end point Limulus Amoebocyte Lysate assay (*Escherichia coli* O55:B5; QCL-1000 Chromogenic endpoint assay kits; Cambrex BioScience, Walkersville, MD, USA) was used to determine the airborne endotoxin and dust endotoxin concentration (endotoxin units/m^3 of air [EU/m^3] and endotoxin units/mg of dust [EU/mg]). The total concentration of endotoxin in the sampled air was calculated in a similar manner as the total dust concentration (Clere and Heart 1994). The assay has a sensitivity range of 0.1 EU/ml to 1.0 EU/ml. Endotoxin samples are referenced to the RSE:EC-6 for conversion to ng/m^3 (10 EU = 1ng). The concentration of endotoxin in a sample is calculated from the values of solutions containing known amounts of endotoxin standard.

4.14. Light microscopy of human lung sections

4.14.1. Histopathology

The lung sections as well as the patients' diagnoses were obtained from Dr. Rani Kanthan from the Department of Pathology, College of Medicine, University of Saskatchewan. These lung tissues were collected from patients who died due to non-infectious pulmonary pathologies (COPD or Asthma) (n=8) and from those who died with no apparent lung pathology (n=6). Five μm thick sections were prepared from the paraffin-embedded human lung tissues and placed on glass slides. These were stained with hematoxylin-eosin.

4.14.2. Immunohistology for human macrophages

After removal of wax with xylene and rehydration in an ethanol series, the sections were incubated in 0.5% hydrogen peroxide in methanol for 20 min to quench endogenous peroxidase followed by pepsin treatment (2 mg/ml 0.01 N HCl) for 45 min for antigen retrieval. After blocking nonspecific sites with 1% bovine serum albumin for 30min, mouse anti-human monocyte/macrophage antibody; MAC 387 (Serotec LTd, Kidlington, UK) at a 1:150 dilution was added to the sections for 60min followed by Polyclonal Goat anti-mouse peroxidase-conjugated secondary antibody (DAKO, Mississauga, ON, Canada) at a 1:150 dilution for 30 min. The antibodies were diluted in 1% bovine serum albumin. The antibody reaction was visualized by use of a color development kit (Vector Laboratories, Burlington, ON, Canada). The slides were counterstained with methyl green (Vector Laboratories, Burlington, ON, Canada). The

negative controls for the immunohistology protocols included staining the lung sections with only secondary antibody and only primary antibody, and the positive control included staining the sections with rabbit anti-human von-Willebrand Factor (vWF) antibody at a 1:300 dilution (DAKO, Mississauga, ON, Canada). Positive septal cells were counted in 40X magnification in 5 randomly chosen fields. The evaluator was blinded to the clinical diagnoses of the samples.

4.15. Statistical analysis

All results were analyzed using computerized statistical software (Statistix 8 Student Edition, Analytical Software, Tallahassee FL 32317, USA). The effect of drug, feed, and drug by feed interaction was analyzed with general Analysis of Variance test with a mare block. Differences between groups were analyzed with an All-Pairwise Comparison Test. Results of the human data were analyzed with a non-paired t-test. Different letters above graphs represent significant differences between the treatments. Differences were considered significant at $P < 0.05$.

5. RESULTS

5.1. Airborne dust and endotoxin concentrations

The mouldy hay (MH) environment contained a higher concentration of dust (3 fold), endotoxin (20 fold), and endotoxin per milligram of dust (7 fold) than the cubes environment (Table 5.1).

5.2. The effect of PIM depletion on the development of clinical signs of heaves

Complete blood cells counts evaluated prior to each BAL were not different among the different treatments. Clinical evaluation performed prior to each BAL revealed a significant higher median of the total clinical scores when mares were fed with MH (MH) compared to Cubes (Cb) (Figure 5.1). When mares were fed with MH and received the GC treatment (MH-GC) the median of the total clinical scores was significantly lower than those without the GC treatment (MH). Gadolinium chloride treatment had no effect on the clinical scores when mares were fed with cubes.

The effects of the different treatments on the five clinical signs chosen to comprise the clinical scores as well as the contribution of each clinical sign to the total clinical scorings are summarized in Table 5.2. While coughing, abdominal effort, auscultation and auscultation after a rebreathing bag were affected by the treatments, nasal flaring was not affected and hence, does not have an effect on the total clinical scores.

5.3. The effect of PIM depletion on the total alveolar cells, alveolar macrophages and neutrophils in the alveolar space in the development of heaves

The total cells concentration in the BALF was significantly higher when mares were fed with mouldy hay (MH) compared to cubes (Cb) (Figure 5.2). When mares were fed with MH and received the GC treatment (MH-GC) the total cell concentration was not statistically different than that in the 3 other treatments. In addition, GC treatment had no effect on the total cell concentration when mares were fed with cubes.

Mares fed with MH had significantly higher concentrations of neutrophils in the BALF compared to when they were fed with cubes (Cb) (Figure 5.3a, 5.3b). When mares were fed with MH and received the GC treatment (MH-GC) the neutrophil concentration was lower than without the GC treatment (MH), and this, as opposed to the total cell concentrations, was statistically significant. Gadolinium chloride treatment had no effect on the neutrophil concentration when mares were fed with cubes.

The different treatments and environments had no effect on the alveolar macrophage concentrations (Figure 5.4).

5.4. The effect of PIM depletion on the activation state of BAL cells

5.4.1. Tumor necrosis factor-alpha (TNFα) protein level and mRNA expression

TNFα protein concentration was assessed using the ELISA method on both BAL supernatants that were obtained immediately following BAL collection (after the first centrifugation, representing the 0 h time point of the different treatments), as well as on cell supernatants that were collected from BAL cells obtained from the different

treatments and incubated with media for 6 h. The assay did not detect TNFα protein in the supernatants collected at the 0 h time point. After 6 h of incubation with media both protein concentration and mRNA expression were not different among the different treatments (Figures 5.5 and 5.6, respectively).

5.4.2. Interleukin-8 (IL-8) mRNA expression

IL-8 mRNA was quantified in BAL cells collected from the different treatment groups after 6 h incubation with media. When mares were fed with mouldy hay (MH) they showed significantly higher expression of IL-8 mRNA than when fed with cubes (Cb) (Figure 5.7). Gadolinium chloride treatment of mares fed with MH (MH-GC) resulted in significantly lower expression of IL-8 mRNA than without the gadolinium chloride treatment (MH). Gadolinium chloride treatment had no effect on the IL-8 mRNA expression when mares were fed with cubes.

5.4.3. Toll like receptor-4 (TLR4) mRNA expression

TLR4 mRNA was quantified in BAL cells collected from the different treatment groups after 6 h incubation with media. When mares were fed with mouldy hay (MH) they showed significantly higher expression of TLR4 mRNA than when fed with cubes (Cb) (Figure 5.8). Mares fed with MH and administered GC (MH-GC) showed significantly lower expression of TLR4 mRNA than without the gadolinium chloride treatment (MH). Gadolinium chloride treatment had no effect on the TLR4 mRNA expression when mares were fed with cubes.

5.5. The effect of PIM depletion on the activation state of BAL cells after a second *in-vitro* challenge with LPS

The ability of BAL cells recovered from different treatment groups to respond to a secondary challenge with LPS was compared. The cells were exposed to LPS *in vitro* for 6 h. The TNFα, IL-8 and TLR4 mRNA expression levels of the media exposure (no LPS) is equivalent to the above results (section 5.4) with an addition of the protein and mRNA expression levels after LPS exposure. Statistical comparisons were preformed between all 8 treatments.

5.5.1. TNFα protein and mRNA expression

The different treatments did not have similar effects on the TNFα protein concentrations as compared to the mRNA expressions. Following exposure to LPS, TNFα protein concentration was significantly lower in cells isolated from mares fed with cubes that received the GC treatment (Cb-GC) compared to the other three treatments, while the mRNA expression was significantly higher in mares fed with cubes (Cb, Cb-GC) compared to MH (MH, MH-GC). Nevertheless, both TNFα protein concentration and mRNA expression were significantly higher following 6 h *in vitro* exposure to LPS compared to media (Figure 5.9 and Figure 5.10 respectively).

5.5.2. Interleukin-8 mRNA expression

Following exposure to secondary hit with LPS, the IL-8 mRNA expression was significantly higher in BAL cells isolated from MH as compared to those isolated from

Cb (Figure 5.11). Moreover, the expression of IL-8 mRNA following LPS exposure was significantly lower in BAL cells isolated from MH-GC, as compared to those isolated from MH. These differences were more pronounced following a second hit with LPS than without the second hit.

5.5.3. TLR4 mRNA expression

TLR4 mRNA expression was not significantly affected by the second hit with LPS (Figure 5.12). In both LPS and non LPS treated cells TLR4 mRNA expression was significantly higher in cells isolated from mares exposed to mouldy hay (MH) as compared to cubes (Cb) and was significantly lower in cells isolated from mares exposed to mouldy hay that received the gadolinium chloride treatment (MH-GC) as compared to without the treatment (MH).

5.6. The presence of septal macrophages in human lungs from healthy and COPD/asthmatic patients

Immunhistology staining of human lung sections revealed no significant differences in the number of MAC387-positive septal cells per field (X40) between healthy and COPD/Ashtmatic patients (Figure 5.13 a,b). However, there was a tendency ($P = 0.10$) for higher number of septal macrophages in the control subjects compared to the COPD/asthmatic subjects.

Subjective evaluation of the same sections stained with hematoxylin and eosin revealed loss of normal pulmonary septal structures and fibrotic changes in some lungs of

COPD/asthmatic patients. In addition some of the sections from the control patients had marked inflammation, edema and pulmonary haemorrhage.

Table 5.1. Airborne dust and endotoxin concentrations and endotoxin content in dust

	Airborne dust concentration (mg/m^3)	Airborne endotoxin concentration (ng/m^3)	Endotoxin content in dust (ng/mg)
Cubes	0.31	0.87	2.8
Mouldy hay	0.94	18.25	19.4

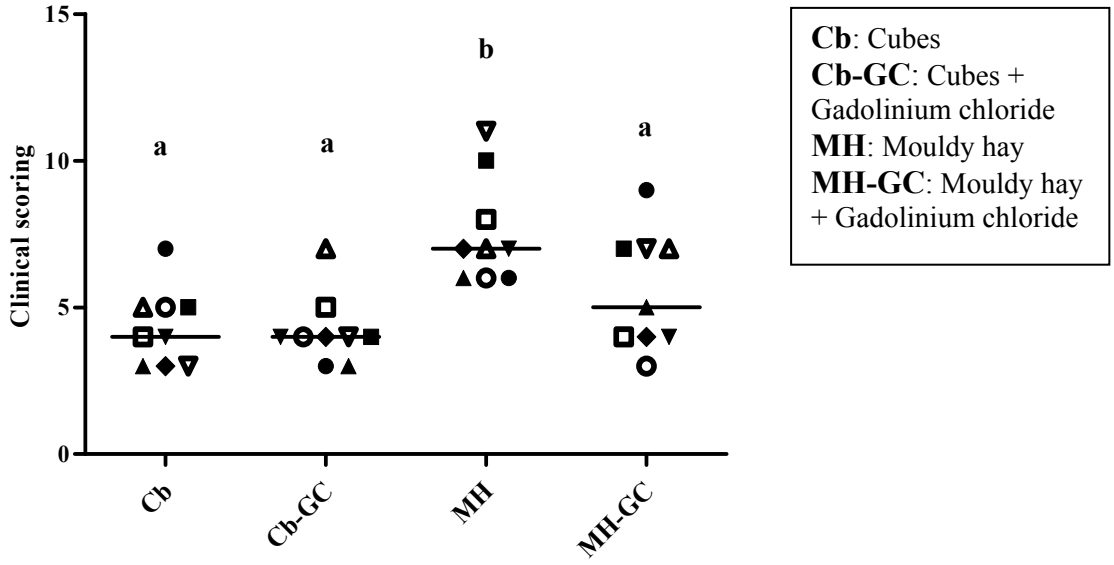

Figure 5.1. Subjective total clinical scores of mares receiving the different treatments. The total scoring was determined by calculating the sum of the abdominal effort, nasal flaring, coughing, auscultation and auscultation after a rebreathing bag. The values represent total clinical score of an individual mare in each treatment; each mare is distinguished by a different shape. Horizontal bars represent the median clinical score of each treatment group. Different letters above represent significant differences between treatments ($P < 0.05$)

Table 5.2. The effects of the different treatments on the scoring of abdominal effort, nasal flaring, coughing, auscultation and auscultation after a rebreathing bag. The effects of Cubes (Cb); Cubes + Gadolinium chloride (Cb-GC); Mouldy hay (MH), Mouldy hay + Gadolinium chloride (MH-GC), on the respiratory effort determined by scores of the following clinical signs (from normal to severe signs): abdominal effort (Abd. Eff. 1-3), nasal flaring (Nas. Flar. 1-3), coughing (0, 1), auscultation (Ausc 1-3) and auscultation after a rebreathing bag (Rebreath 1-3).

		Actual sum of mares			
Clinical sign	Possible range (sum of mares)	Cb	Cb-GC	MH	MH-GC
Abd. Eff. (1-3)	9---27	11	10	21	14
Nas. Flar. (1-3)	9---27	10	9	10	9
Coughing (0,1)	0---9	0	0	4	2
Ausc. (1-3)	9---27	10	10	20	15
Rebreath. (1-3)	9---27	10	9	20	15

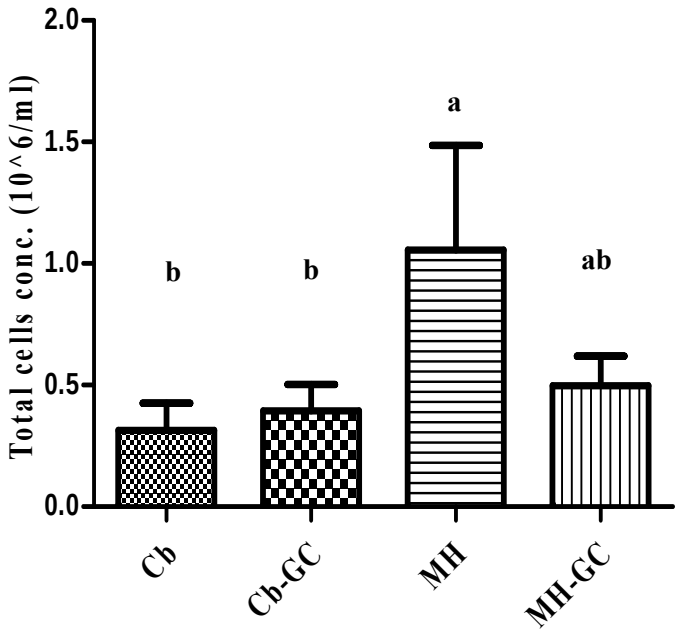

Figure 5.2: Total cell concentration (mean) in the BALF collected from mares in the different treatments. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

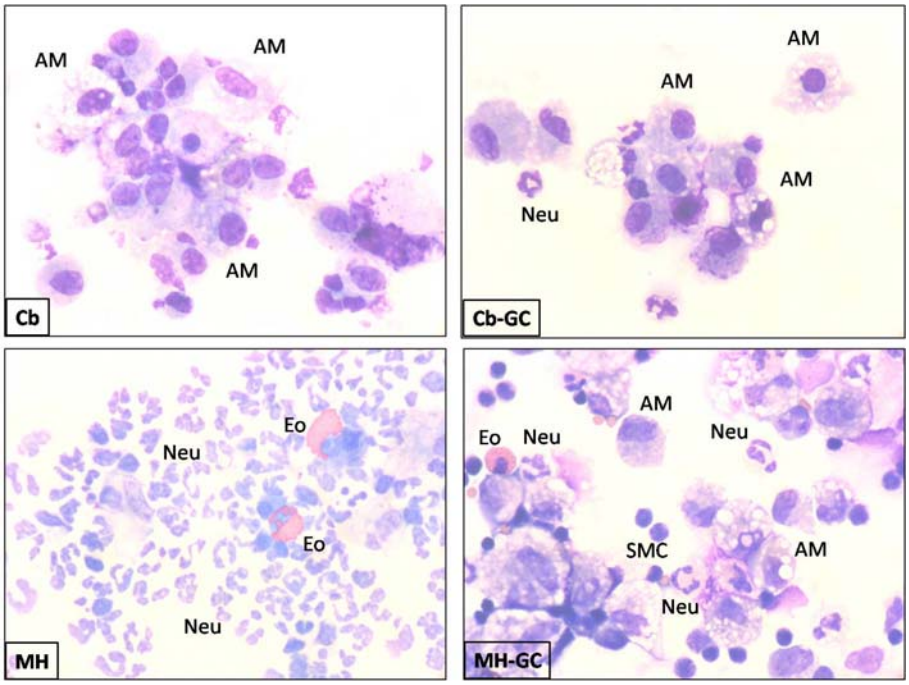

Figure 5.3a A cytospin of BAL from one representative mare which received the 4 treatments: Cubes (Cb); Cubes + Gadolinium chloride (Cb-GC); Mouldy hay (MH), Mouldy hay + Gadolinium chloride (MH-GC). Alveolar macrophages – AM, Neutrophils – Neu, Eosinophils – Eo, Small mononuclear cells – SMC. X40

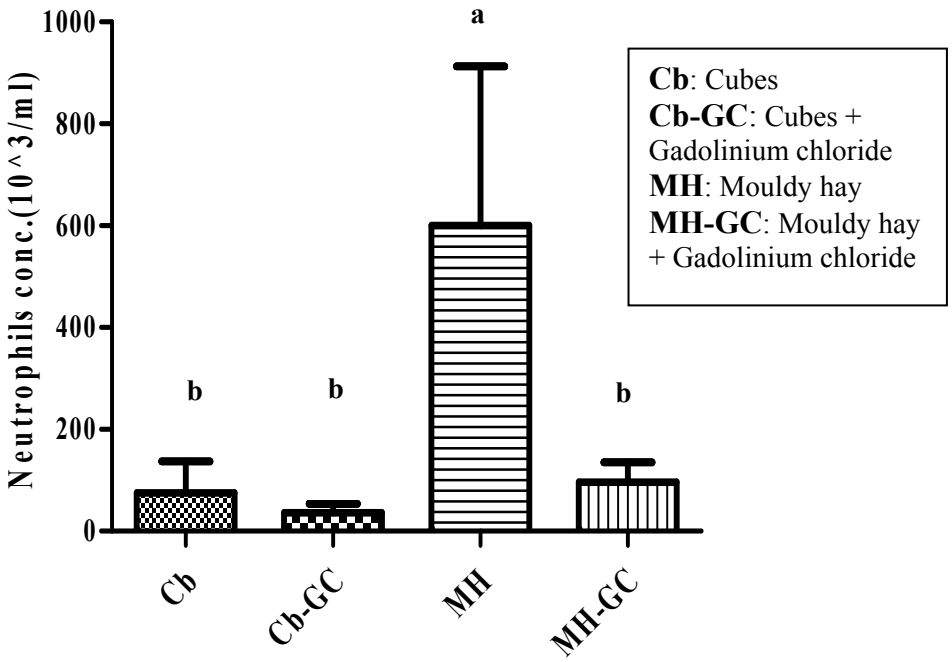

Figure 5.3b Neutrophil concentration (mean) in the BALF collected from mares in the different treatments. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

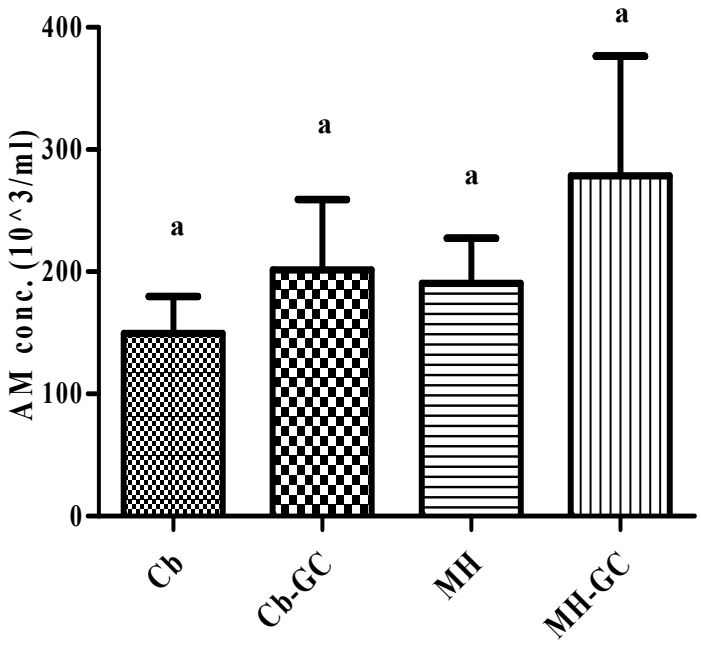

Figure 5.4. Alveolar macrophage concentration (mean) in the BALF collected from the different treatment groups. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

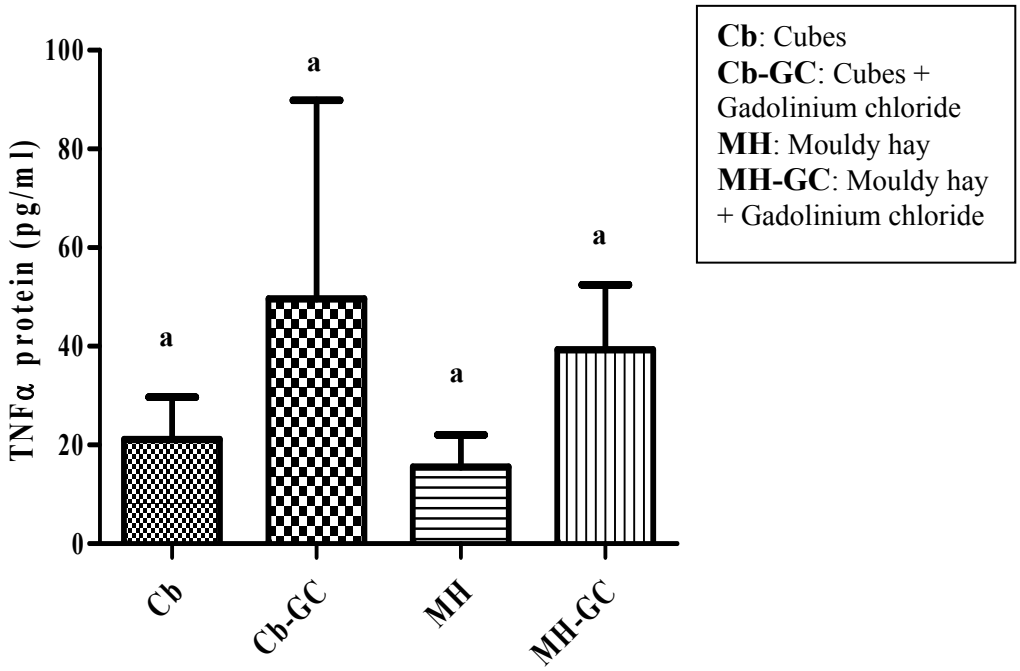

Figure 5.5. ELISA results (mean) for the TNFα concentration in BAL cells recovered from the different treatments. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

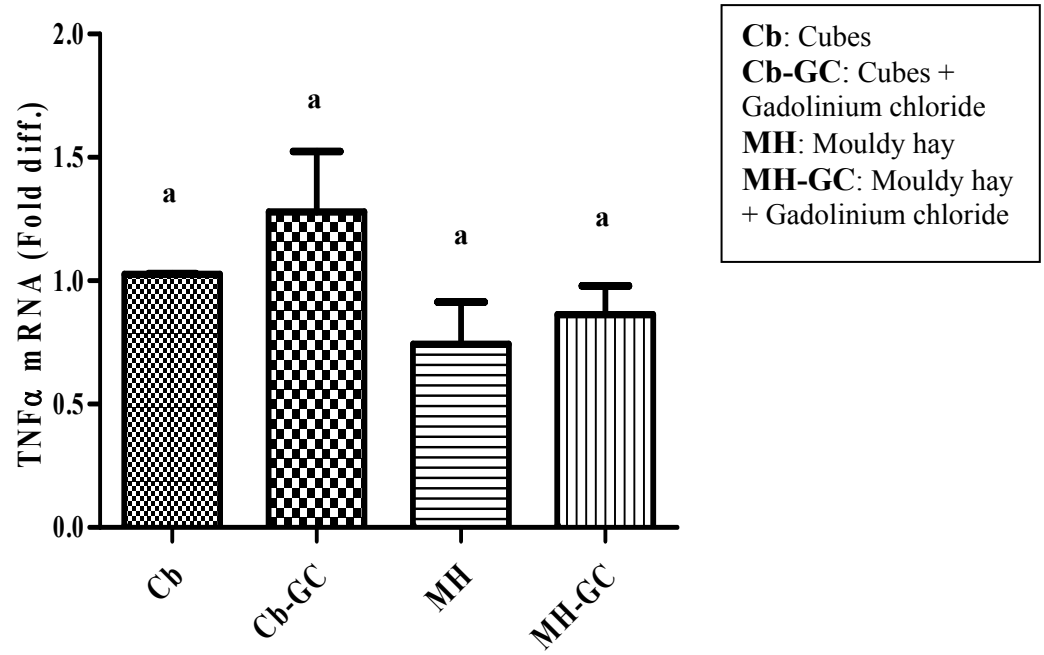

Figure 5.6. Real time PCR results (mean) of the fold difference in TNFα mRNA expression from BAL cells recovered from mares in the different treatments. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

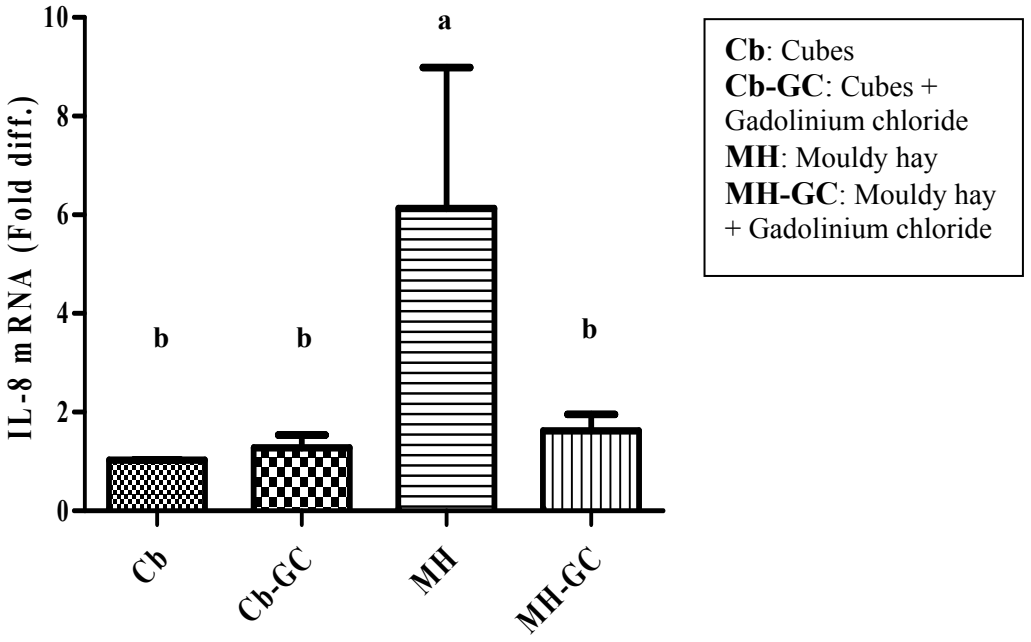

Figure 5.7. Real time PCR results (mean) of the fold difference in IL-8 mRNA expression from BAL cells recovered from mares in the different treatments. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

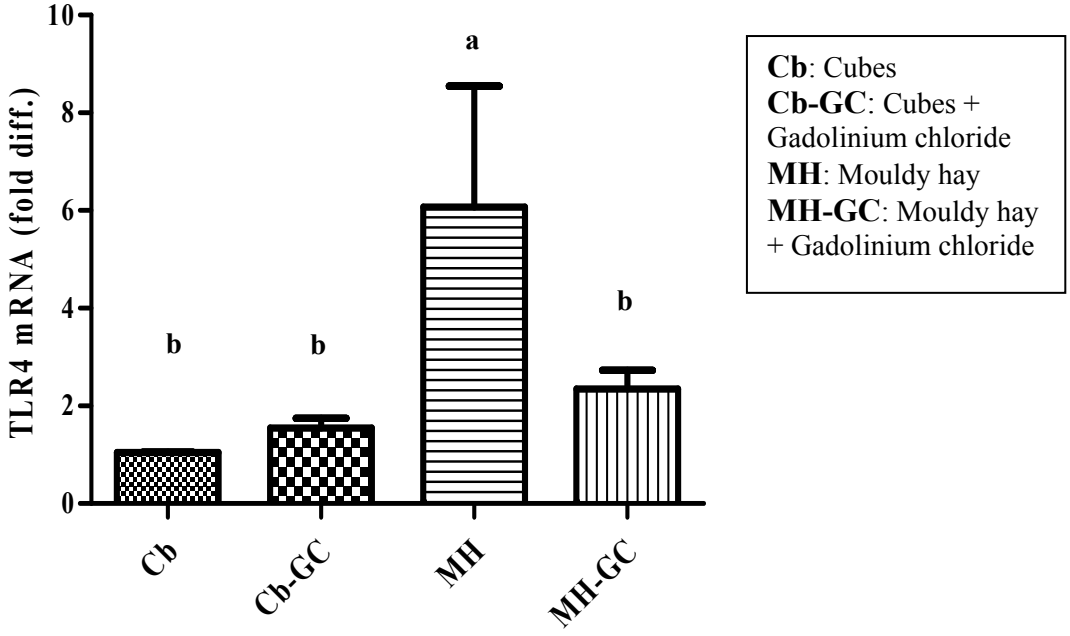

Figure 5.8. Real time PCR results (mean) of the fold difference in TLR4 mRNA expression from BAL cells recovered from mare in the different treatments. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

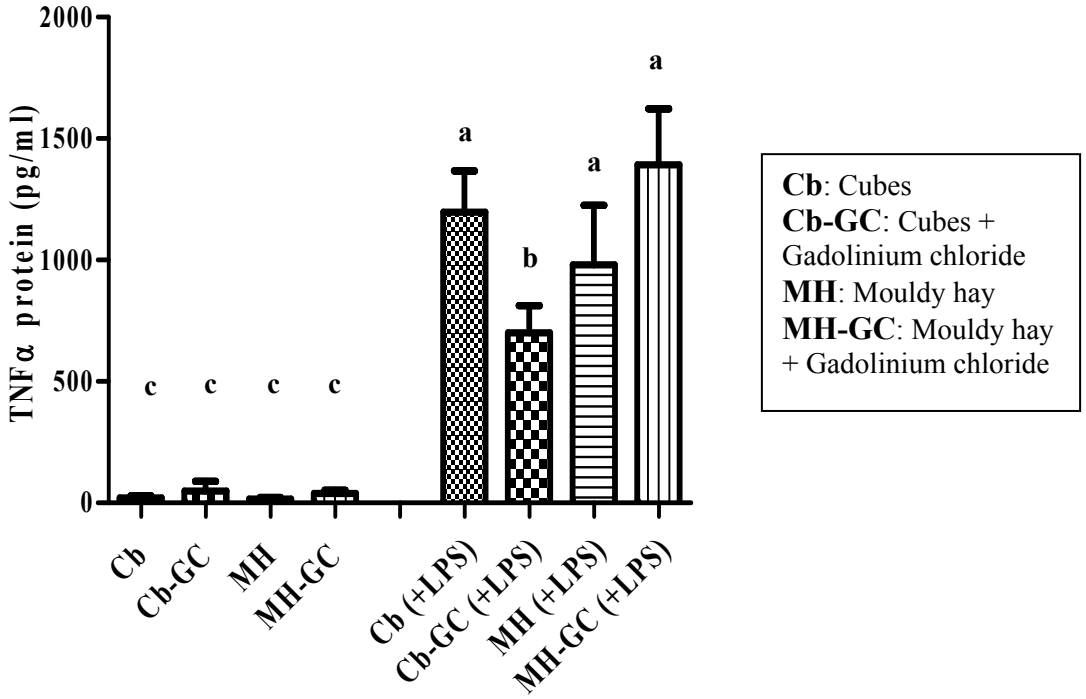

Figure 5.9. ELISA results (mean) of the TNFα concentration from BAL cells recovered from mares in the different treatments after 6 h exposure to LPS or media. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

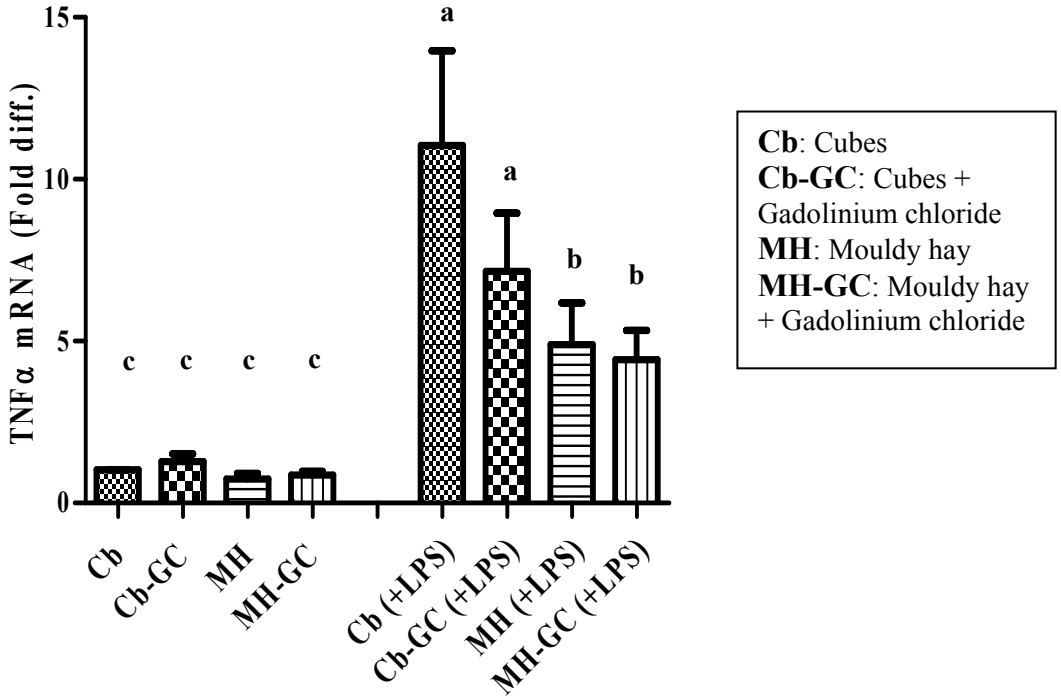

Figure 5.10. Real time PCR results (mean) of the fold difference in TNFα mRNA expression from BAL cells recovered from mares in the different treatments after 6 h exposure to LPS or media. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

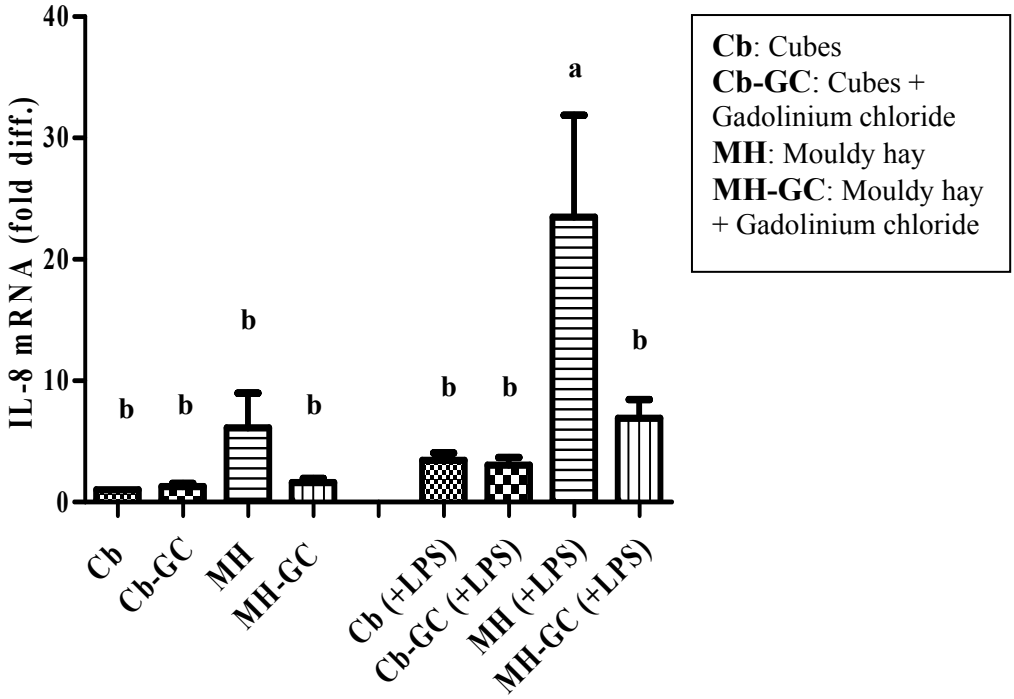

Figure 5.11. Real time PCR results (mean) of the fold difference in IL-8 mRNA expression from BAL cells recovered from mares in the different treatments after 6 h exposure to LPS or media. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

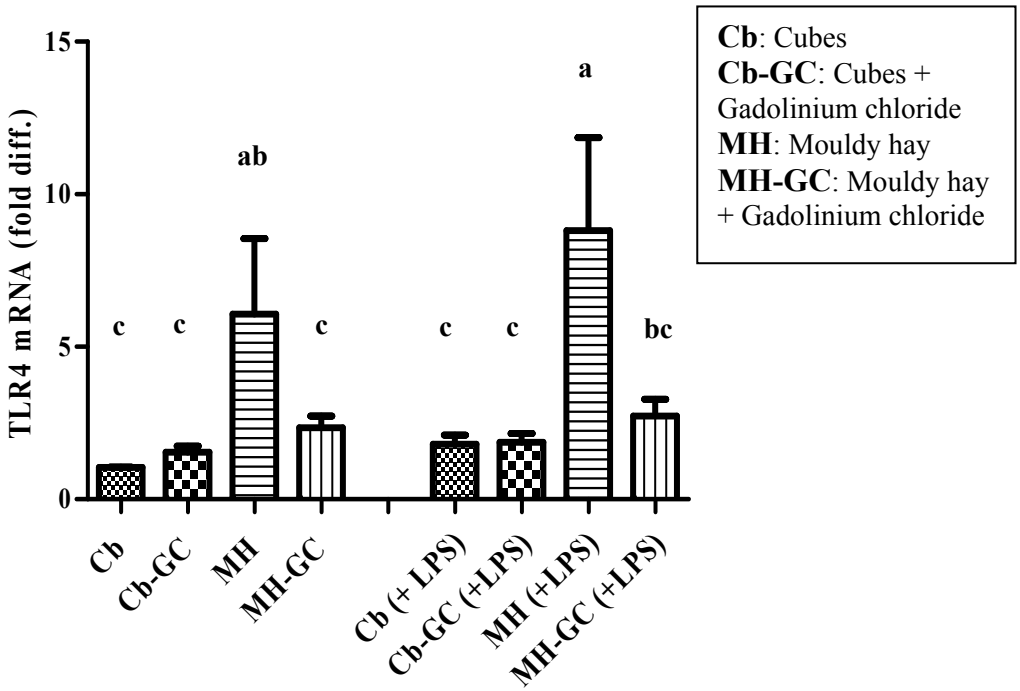

Figure 5.12. Real time PCR results (mean) of the fold difference in TLR4 mRNA expression from BAL cells recovered from mares in the different treatments after 6 h exposure to LPS or media. Error bars represent S.E.M. Different letters above bars represent significant differences between treatments ($P < 0.05$)

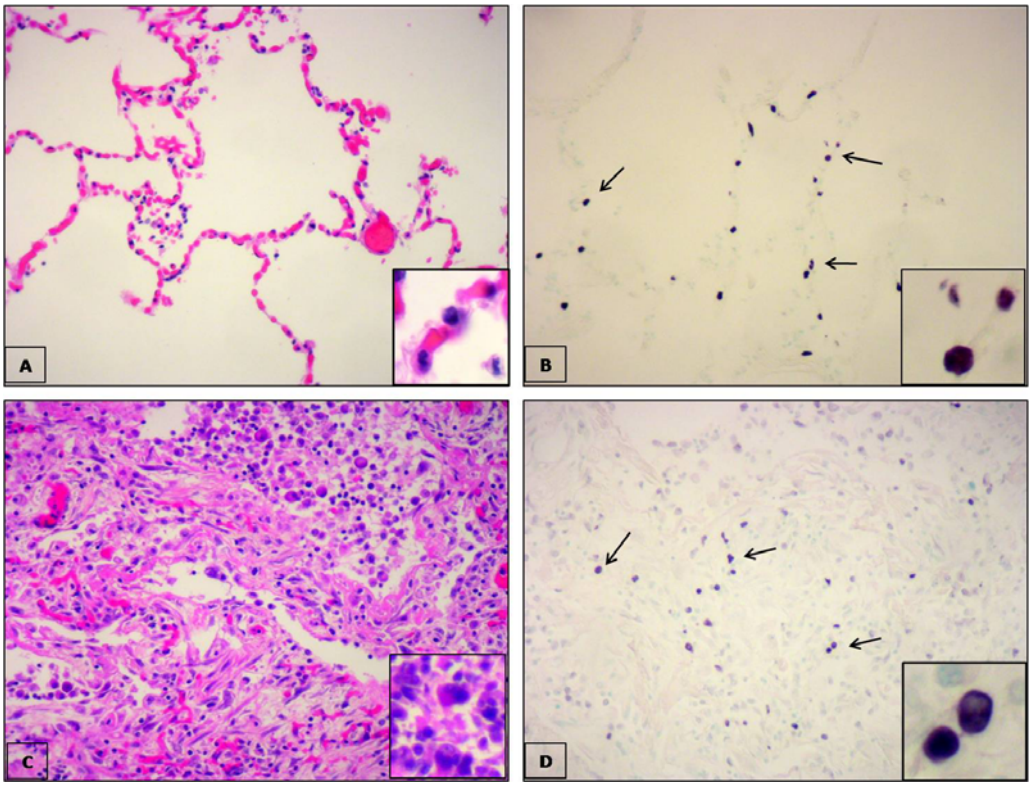

Figure 5.13a Representative histology stained with hematoxylin-eosin (H&E) (A,C) and immunohistochemistry for MAC387 (B,D) in human lung sections from control (A,B) and COPD/asthmatic subjects (C,D). Arrows and inserts represent septal macrophages. Original magnification: X10; inserts: X40

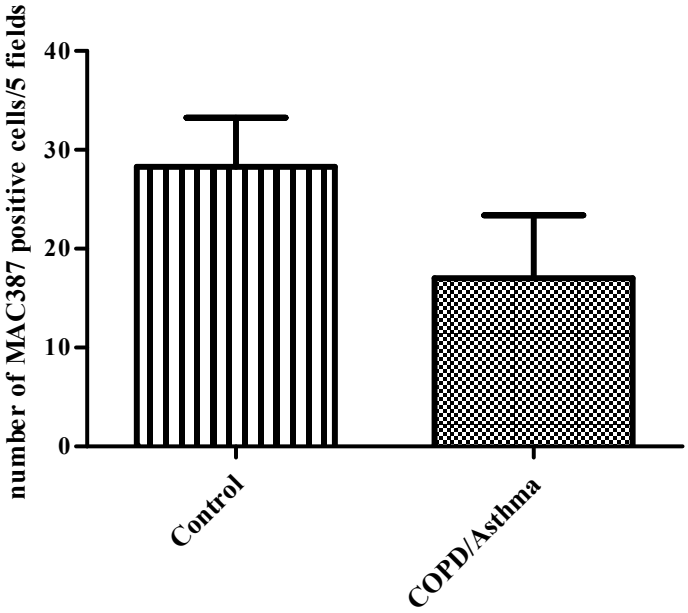

Figure 5.13b Numerical quantification (mean of counts in 5 fields of X40) of septal cells positive for MAC387 in human lung sections from control (n=6) and COPD/Asthmatic patients (n=8). Error bars represent S.E.M.

6. DISCUSSION

The role of PIMs in the development of acute lung injury following endotoxemia has previously been addressed in different animal species. Horses were shown to contain resident PIMs and PIM depletion attenuated acute lung injury after intravenous administration of endotoxin. However, the role of these unique macrophages in the development of lung pathologies such as heaves, in which the pathogenic agent is introduced via the airways, remains to be investigated. I compared the development of heaves in normal and PIM-depleted horses to investigate the inflammatory role of these cells in the pathophysiology of equine heaves.

6.1. Validation of the study design

In order to investigate the involvement of PIMs in the pathogenesis of heaves, the first task was to be able to induce heaves in susceptible horses and to be able to achieve clinical remission. It is well documented that heaves is triggered by a dusty environment and it has been suggested that endotoxin in the dust is a major contributor to the development of this pathology (Pirie *et al.* 2001; Davis and Rush 2002; Pirie *et al.* 2003a; Pirie *et al.* 2003b). I introduced a "dusty" environment by feeding the horses with two year-old mouldy hay in round bales. The "clean" environment (low-dust) was achieved by feeding the horses with alfalfa cubes. The analysis of the dust and endotoxin collection revealed a higher concentration of airborne dust and endotoxin and higher concentration of endotoxin in dust in the MH environment than in the cubes environment. These findings are in accordance with a previous report (McGorum *et al.* 1998) which measured the amount of dust and endotoxin in conventional stables and in pasture; the

MH environment in the current experiment contained slightly lower levels of airborne dust and airborne endotoxin than was measured in conventional stables in the above cited report (0.94 vs. 2.74 mg/m^3, 18.2 vs. 19.76 ng/m^3, respectively), but more endotoxin content in the dust (19.4 vs. 7.57 ng/mg). The alfalfa cubes environment in our study contained less endotoxin content in dust than was reported for the pasture environment (2.8 vs. 4.85 ng/mg) which is considered, to date, the ideal environment to achieve remission from heaves. These findings are not surprising. The mares in this study were fed in outdoor paddocks, therefore the total dust and endotoxin in the air is likely to be lower than what is found in indoor stables. However, the hay was of poor quality and probably older and mouldier than hay in most conventional stables, which may have resulted in higher amount of endotoxin per milligram of dust in the current experiment. Hay given in round bales encourages the horses to bury their whole head in the hay, therefore, not only did the MH contain more dust and endotoxin than the cubes, but this eating pattern exposes the horses to higher levels of dust and endotoxin (Figure 4.1). Evaluation of the dust and endotoxin concentrations was performed in order to determine whether the environments introduced by us were able to mimic those previously reported to induce heaves and remission from heaves. Since this was not the main objective of this study, the collection procedure was performed only once, and compared to past publications. Other than the endotoxin, several components of the hay dust such as moulds (Simonen-Jokinen *et al.* 2005) were also shown to contribute to the development of heaves. Assessment of the levels of these components in our environmental settings would have increased the validity of our study design. However, since endotoxin concentrations in stable dust were previously shown to exceed those causing symptoms in

man (McGorum *et al.* 1998) and endotoxin inhalation has been reported to elevate BALF neutrophil counts and MMP-9 levels in both heaves susceptible and healthy horses (Pirie *et al.* 2001; Nevalainen *et al.* 2002), I decided to focus on the endotoxin and dust concentrations.

In this study design, mares were exposed to MH for 7 days to induce heaves, and thereafter were fed alfalfa cubes for 21 days to achieve remission. There are variations in the reports regarding the length of time horses should be exposed to a dusty environment to induce clinical signs of heaves (Leguillette 2003) and the length of time in which they should be in pasture to achieve clinical remission (Davis and Rush 2002; Couetil and Ward 2003; Leguillette 2003). This discrepancy is probably due to the chronic or recurrent nature of the disease. Horses that had previous exposure to a dusty environment and experienced heaves during their lifetime would be more susceptible and hence rapidly develop clinical signs. Furthermore, in addition to the type of food (Robinson *et al.* 2006), other environmental factors have been suggested to effect the development of heaves; such as bedding (straw vs. wood shavings) (Couetil and Ward 2003), ventilation quality (Couetil and Ward 2003), outdoor/indoor facilities (Tremblay *et al.* 1993; Dixon *et al.* 1995) and weather (dry/humid, winter/summer) (Bowles *et al.* 2002). There is also great variability between individual horses with differences in susceptibility between horses (Laan *et al.* 2006). In an attempt to overcome this, my experiment was designed as a crossover study in which all horses ultimately received all treatments and the data was analyzed statistically by blocking the effect of the mare. Although a crossover design has the disadvantages of doubling the duration of the study as well as the carryover effects, it minimizes the potential for confounding factors, such as discussed above, since each

participant serves as his own control and substantially increases the statistical power of the trial so that it needs less participants (Hulley *et al.* 2001). The mares in my study had past exposure to dusty environment and a history of heaves. Therefore, they were considered susceptible to heaves. Feeding these susceptible mares with MH for a period of 7 days was sufficient to induce clinical signs of heaves, as indicated by the clinical scoring analysis. Several studies reported an improvement of the respiratory function of heaves horses 1 week after being turned outside on pasture (Leguillette 2003), while others showed remission of signs only after four to six weeks of putting horses in controlled environment or pasture (McGorum *et al.* 1993a; Vandenput *et al.* 1998; Davis and Rush 2002). In order to prove remission from heaves, assessment of the clinical signs is not sufficient and further tests should be performed such as BAL and intrapulmonary pressure (ΔPpl) analyses. While we did not confirm remission of heaves with BAL or other relevant tests, mares in this study were fed with alfalfa cubes for a period of 3 weeks to achieve remission. It is possible that the mares did not achieve complete remission which would affect the results of the "non-heaves induced mares" by bringing them closer to the results observed in the "heaves-induced mares"; this further strengthens the significant differences that were found in this study.

6.2. Depletion of PIMs attenuates the development of heaves

Different agents such as clodronate (Sone *et al.* 1997), and tyloxapol (Staub *et al.* 2001) have been used in research to inactivate or deplete the PIMs. Gadolinium chloride was shown to kill and eliminate macrophages as well as interfere with their phagocytic function (Mizgerd *et al.* 1996). It is believed that GC leaks into the cytoplasm following

its endocytosis and kills macrophages either by direct cell injury or apoptosis (Singh and de la Concha-Bermejillo 1998). Because phagocytosis of GC is a prerequisite for its efficacy, macrophages become its prime targets (Singh *et al.* 2004). The macrophages affected most by intravenous GC injection *in vivo* are those that reside within the vasculature (Kupffer cells, splenic macrophages and PIMs). In species having PIMs, most of the biological or other particulate material injected via jugular vein is removed by the PIMs due to the "first pass" in the lung. Consequently there may be little spill over of GC to Kupffer cells thus reducing the possibility of damage to these cells (DeCamp *et al.* 1992; Staub 1994; Singh and de la Concha-Bermejillo 1998). However, the effect of intravenous administration of GC on the alveolar and interstitial macrophages is not well established because there are no data on the transport of GC across the blood-air barrier. If GC indeed leaks from the vasculature into the alveolar space and the interstitium, the outcomes seen in the current study could be partially due to the elimination of the alveolar or interstitial macrophages. It was previously shown that a single intravenous administration of GC did not affect alveolar or interstitial macrophages in rats (Bannenberg *et al.* 1995). However, multiple GC treatments (36 weekly injections) in lambs resulted in ultrastructural observations of GC-like material in endosomes of alveolar macrophages along with some signs of damage suggestive of apoptosis (Singh and de la Concha-Bermejillo 1998). Although the mares in my study received a total of four treatments with GC (one while exposed to cubes and three while exposed to MH), the alveolar macrophage concentrations were not affected by the treatments nor was there an increase in number of apoptotic cells observed on cytospin slides from BALF of mares

treated with GC. These findings suggest lack of effect of GC on alveolar macrophages in this study.

The effect of GC on other cells in the lungs possessing phagocytic capabilities, such as neutrophils, dendritic cells, and endothelial cells was not assessed in this study. The consequences of inhibiting or depleting such cells would be similar to those seen in our study (i.e. attenuation of the inflammatory processes), due to a decrease in the chemotactic gradient and decrease in adhesion complexes expressed on the endothelium. The protocol used to deplete the PIMs in this study was similar to a previously reported protocol which showed the effect of intravenous administration of GC on the depletion of PIMs in horses (Parbhakar *et al.* 2004). In the above cited study, a continuous decline of PIMs was observed at 72 h after GC administration. In order to avoid a possibility of insufficient clearance of the GC by the PIMs and consequently its leakage and effect on Kupffer cells in the liver, I administered GC every 96 h. Since we did not rule out the effect of GC on other cells in the lung that could attribute to the inflammatory process, we must take caution in ascribing the outcomes of this study solely to the depletion of the PIMs. However, due to the avid phagocytic ability of macrophages, the effect of GC on other cells might be of less significance.

The main objectives in this study were to determine whether depletion of the PIMs would attenuate the development of heaves in horses. The investigator performing the clinical examinations was not blinded to the treatments, which might raise the concern of biased results; nevertheless, the rest of the experiments (cells counts, protein and mRNA levels) were carried out by an investigator blinded to the treatments. Therefore, although the issue of bias may have played a role on the clinical assessment of

horses, it may not be of significance as the cellular and molecular results were parallel to the clinical scores.

While exposure to MH resulted in higher clinical scores, GC-induced PIM depletion decreased the median clinical scores in heaves horses. The clinical signs chosen to compose the clinical scoring in this experiment were abdominal effort, nasal flaring, coughing, auscultation and auscultation after a rebreathing bag. These were not affected equally by the treatments. Robinson and colleagues reported that coughing cannot be assessed accurately by counting during brief periods due to its sporadic nature (Robinson *et al.* 2003). However, I found this clinical sign to be well affected by the different treatments and hence was an important addition to the total clinical scoring. Nasal flaring is often used in diagnosis and in clinical scoring systems to determine the severity of heaves (Rush *et al.* 1998b; Couetil *et al.* 2001; Courouce-Malblanc *et al.* 2007), but in the current study nasal flaring was hardly noticeable and did not seem to be affected by the different treatments and therefore did not effect the total clinical scores. There are several discrepancies between studies regarding the clinical signs used to determine the severity of heaves and a standardised, internationally accepted clinical scoring system for equine lower airway disease is required.

Similar to observations of other investigators, I also did not find any alterations in hemograms of mares following induction of heaves (McPherson *et al.* 1978; Davis and Rush 2002), and following the GC treatments (Parbhakar *et al.* 2004).

The clinical signs of heaves are due to bronchoconstriction and accumulation of mucus and neutrophils in the airways (Robinson *et al.* 1996; Robinson *et al.* 2003). Indeed, mares exposed to MH showed an increase in the total cell and neutrophil

concentrations in the BALF. Depletion of PIMs reduced the neutrophil concentration to the control levels. It is well documented that activation of PIMs by intravascular LPS enhances the migration of neutrophils into the lung (Warner 1996; Singh *et al.* 2004; Parbhakar *et al.* 2005). However, this is the first study of the effect of inhaled endotoxin as well as other irritating molecules on the equine PIMs that are embedded in lung vascular endothelium. The results of this study indicate a potential role of the PIMs in the development of an airborne pathology. The PIMs are located on the vascular side of the air-blood barrier, thus their involvement in an airborne pathology is intriguing. One explanation for such involvement of PIMs could be transport of an airborne pathogen or infectious agent across the blood-air barrier, either through transcellular routes across an intact barrier or following physical disruption of the barrier leading to their interaction with PIMs. It was previously shown in cattle that intratracheally instilled *M. hemolytica* leukotoxin was detected in PIMs which suggests leukotoxin transfer across the blood-air barrier (Whiteley *et al.* 1990). The TLR4 expressed in equine PIMs will enable them to respond to endotoxin transported across the blood-air barrier (Singh Suri *et al.* 2006). Another explanation is that the inflammatory response generates circulating particulate debris and cells that are cleared by the PIMs which activates them with or without the transport of the infectious agents across the air-blood barrier (Warner 1996). It is also possible that inflammatory mediators produced by activated airway epithelium and alveolar macrophages in heaves horses may activate PIMs either following migration across the blood-air barrier or through production of an intermediary signalling molecule from the alveolar epithelium. Similar mechanisms have been reported in rats following instillation of TNFα in the alveoli (Kuebler *et al.* 2000). Although the mechanism

remains unclear, the data presented here showing reduced lung inflammation in GC-treated horses indicate activation of PIMs and their pro-inflammatory role in heaves.

The mode by which the depletion of PIMs reduces the neutrophil concentration in the alveolar space of horses with heaves can be explained in several ways. PIMs were shown to contain TLR4 and to be the major source of TLR4 in the lungs of horses (Singh Suri *et al.* 2006). Hence, LPS-LBP-CD14 complex associates with TLR4 and results in activation of the PIMs. Consequently, these activated PIMs secrete pro-inflammatory mediators (Parbhakar *et al.* 2005) that promote pulmonary recruitment of neutrophils. In addition, the increase in neutrophil concentration in the alveolar space of horses with heaves is believed not only to be due to increased migration of these cells, but also to the delay in their apoptosis. This enhanced neutrophils survival is induced, among others, by granulocyte/macrophage colony stimulating factor (GM-CSF) (Turlej *et al.* 2001).

The significant increase in TLR4 mRNA when mares were fed with MH can imply either that TLR4 expression is increased or that the proportion of cells expressing TLR4 has increased in the BALF. Regardless of the mechanism, the increase in TLR4, a crucial receptor of LPS (Aderem and Ulevitch 2000; Baumgarten *et al.* 2006), marks the increase in an inflammatory response which at least in part is triggered by endotoxin in MH. This result is in agreement with work done by Berndt et al. which demonstrated an involvement of TLR4 in the pathogenesis of heaves by showing an increase in TLR4 mRNA expression in bronchial epithelial cells from heaves horses exposed to stable dust. This increase in TLR4 mRNA correlated with IL-8 mRNA expression as well as with neutrophilic airway inflammation (Berndt *et al.* 2007).

In addition to the lung cells such as pulmonary epithelial cells (Monick *et al.* 2003; Ainsworth *et al.* 2006), alveolar macrophages (Fernandez *et al.* 2004; Singh Suri *et al.* 2006), endothelial cells (Andonegui *et al.* 2003; Singh Suri *et al.* 2006), airway smooth muscle cells (Morris *et al.* 2005), and PIMs (Singh Suri *et al.* 2006) that contain TLR4 on their surface, neutrophils were also shown to express this receptor (Sabroe *et al.* 2002; Marsik *et al.* 2003). Activation of TLR4 on neutrophils causes changes in adhesion molecules expression, respiratory burst and IL-8 generation. Furthermore, the prolonged neutrophil survival which is a central component of their responses to pathogens, and as mentioned previously occurs in BAL neutrophils of heaves horses, was shown to require both TLR4 activation and the presence of monocytes which upon TLR4 activation release survival factors (Sabroe *et al.* 2003). The fact that GC treatment reduced the TLR4 expression in BALF cells almost back to the control (Cb) implies that the depletion of PIMs not only inhibits the number of inflammatory cells that migrate to the airspace but also lowers the susceptibility of cells that do migrate to the alveolar space to LPS and consequently lowers the inflammatory response.

6.3. Depletion of PIMs protects against a second endotoxin challenge

Horses with severe airway obstruction may develop secondary bacterial infection, due to lower clearance capability (Davis and Rush 2002), although once the secondary pathogen enters the small airways it may be eliminated faster due to increased number of activated inflammatory cells in the airways and an increased inflammatory response. Other than the possible opportunistic infections that may occur, the ongoing exposure to dust and endotoxin in the dust in horses with heaves ultimately generates an exaggerated

inflammatory response leading to worse clinical signs and prognosis of the horse. Hence, I raised the question whether cells recovered from BALF of horses with heaves behave differently to a second LPS challenge when mares were pre-treated with GC. Although the mRNA expression levels of the 6 h media exposure are equivalent to the results in the previous section, the statistical significances may be different than reported in the earlier section since more values were included in current statistical analysis (i.e. combined analysis on both LPS and no-LPS results vs. analysis only on the non-LPS results).

It was previously shown that a strong chemotactic activity in the BALF is associated with high levels of dust exposure and that *in vitro* stimulated alveolar macrophages have impaired phagocytotic efficiency but become specialized in secretion of neutrophils chemo-attractants such as IL-8 and macrophage inflammatory protein-2 (MIP-2) (Franchini *et al.* 1998). In the study presented here, a significant increase in the IL-8 mRNA expression was demonstrated in cells isolated from mares after MH exposure. This increase was more pronounced after challenging the cells with a "second *in vitro* hit" with LPS. The IL-8 mRNA expression decreased almost back to the control levels (Cb) in cells isolated from mares exposed to MH and treated with GC. In addition, cells recovered from mares that were exposed to MH and treated with GC did not react to the second LPS hit, or reacted similar to the cells recovered from the control environment (Cb). Hence, it appears that depletion of PIMs by GC either inhibits the expression of chemotactic factors by inflammatory cells that reside in the bronchoalveolar space, or inhibits the migration of cells responsible to such chemotactic activity, leaving only the inactivated resident cells in the bronchoalveolar space. Nevertheless, the results presented here are in agreement with other studies which showed that an ongoing exposure to LPS

such as often occurs in heaves, or a secondary bacterial infection in heaves horses, causes an increase in the chemotactic activity in the bronchoalveolar space (Franchini *et al.* 1998). This chemotaxis may be reduced to the control levels by depletion of the PIMs which will eliminate a major source of proinflammatory cytokines. These proinflammatory signals normally activate inflammatory cells in the airways or activate endothelium and inflammatory cells in the vasculature to migrate to the airway and continue to produce pro-inflammatory chemokines and cytokines.

As opposed to IL-8, TLR4 mRNA expression did not seem to be affected by the second *in vitro* LPS challenge. It was previously shown that both the dose of LPS as well as the length of exposure determines the level of TLR4 expression in macrophages cells lines. A high dose of LPS (100 ng/ml) for 2.5 h severely reduced the mRNA expression of TLR4; however, the expression returned to the original level after a 20 h stimulation. In addition, TLR4 mRNA expression level at 24 h of LPS treatment was almost at the same level as that of non-treated cells (Nomura *et al.* 2000). The LPS dose used in the current study was 1 μg/ml; a dose higher than the "high dose" of the above cited report. Hence, it is possible that an overstimulation of the cells in this study initially caused a shutdown or down-regulation in the TLR4 expression and after 6 h exposure to LPS the TLR4 expression was restored back to its original levels (i.e. without the LPS).

TNFα protein was not detected in the supernatant collected immediately after the BAL procedure. This is probably due to the high volumes of lavage fluid used (1 liter for each horse), which exceedingly diluted the sample. Inconsistencies between the TNFα protein concentrations and the mRNA expression levels after incubation of the cells were found. The cause of the inconsistencies is not apparent from the results. In addition the

results did not show any consistent or meaningful effect of the different treatments (i.e. mouldy hay, cubes and gadolinium chloride) on the TNFα expression. However, a significant increase in the TNFα protein and mRNA expression was induced by a second *in vitro* LPS exposure. These results are not consistent with a previous report which indicated an increase in the TNFα mRNA in BALF of horses with heaves after exposure to MH (Giguere *et al.* 2002). Nevertheless, most of the investigations concerning cytokine production in horses with heaves concentrated on the question whether the response is predominately Th2 (i.e. IL4 and IL5) or Th1 (i.e. INF-γ) and no consistent findings on cytokine mRNA expressions were found between studies (Giguere *et al.* 2002; Cordeau *et al.* 2004; Horohov *et al.* 2005). These disparate results were suggested to be the result of differences in the clinical stage of the affected animals or the timing of sample collection. Horses in the earliest stage of heaves are likely to exhibit a classic Type 2 cytokine response to the allergen but prolonged exposure to the allergen induces a chronic phase characterised by increased production of IL-8 and IFN- γ with subsequent airway remodelling (Horohov *et al.* 2005). TNFα is an acute phase cytokine and it is possible that lack of differences for this cytokine among groups may be due the stage of disease in the experimental animals. However, a second hit with LPS initiated an acute response marked by a significant increase in the protein and mRNA levels of TNFα indicating that cells were capable of producing the cytokine and that my results were not due to any technical issues.

In order to minimize irritation during the BAL procedure which may lead to coughing and consequently damage the bronchi, 2% lidocaine HCl was injected to the airways while passing the endoscope. The anti-inflammatory effect attributed to lidocaine

(Kawasaki *et al.* 2006; Feng *et al.* 2008) could have affected the results observed in cells retrieved from the MH challenge as well as the second *in-vitro* LPS challenge. Since the amount used in this study (20 ml) was greatly diluted with the 500 ml of saline used to flush each side of the lung, and since the same procedure was carried out in all the BALs performed in all the mares, the effect of lidocaine is most likely minor in this study.

The data from the horse experiments are novel since they identify the PIMs as important cells involved in the development of lung pathology such as heaves, which is induced by inhaled irritants. Following exposure to the dust and endotoxin in the MH environment the mares developed clinical signs suggestive of heaves as well as increased concentration of total bronchoalveolar cells, mainly neutrophils. This increase in neutrophil concentration in the bronchoalveolar space coincides with the increase in neutrophil chemoattractant (IL-8) and LPS recognition receptor (TLR4). I suggest a pro-inflammatory affect of the PIMs, since after their depletion the clinical development as well as the accumulation of inflammatory cells and pro-inflammatory molecules subside. The depletion of the PIMs not only protected from the development of heaves but also from an exaggerated inflammatory response following a second challenge with LPS. Figure 6.1 outlines the major findings of this study.

6.4. PIMs in human lungs

There is a controversy surrounding the occurrence of PIMs in human. Dehring and Wismar (Zeltner et al. 1987; Dehring and Wismar 1989) showed large mononuclear cells with phagocytic vacuoles in clinical human lung biopsy specimens, but morphometric studies of normal human lungs have not described macrophages or

unusually large mononuclear cells in pulmonary capillaries (Gehr et al. 1978; Zeltner et al. 1987). The accepted notion today is that the normal human lung does not appear to have a large population of phagocytically active mature macrophages in pulmonary capillaries. However, it is known that rats can recruit PIMs under physiological stress induced with sepsis (Charavaryamath et al. 2006) or liver damage (Chang and Ohara 1994). Based on the data obtained from the horses on the role of PIMs in an airborne non-infectious lung pathology, I decided to examine whether there is recruitment of PIMs in lungs of humans suffering from COPD or asthma.

Equine heaves resembles several human pulmonary diseases, mostly asthma but also COPD, both in the pathogenesis of the diseases and in the contribution of endotoxin to the development and exacerbation of the diseases. Inhaled endotoxins are an important cause of human COPD (Rushton 2007), with the severity of pulmonary inflammation and clinical symptoms experienced by subjects exposed to organic dusts being related to the endotoxin concentration of the inhaled dust (Rylander and Bergstrom 1993; Smid *et al.* 1994; Iversen *et al.* 2000; Kirychuk *et al.* 2006). In addition, the severity of human asthma has also been related to the level of endotoxin exposure, suggesting that inhaled endotoxin may potentiate the inflammatory response to allergens in atopic subjects (Michel *et al.* 1996; Rizzo *et al.* 1997). If indeed humans recruit PIMs under these pathological conditions, depletion of these cells, similar to the horses, might ameliorate the clinical development of these diseases.

The immunhistology results on the number of pulmonary septal macrophages did not reveal a significant difference in humans COPD/asthmatic patients as compared to the control patients. Surprisingly, there was a tendency ($P = 0.1$) of lower number of septal

macrophages in the diseased patients than in the control, which suggests occurrence of higher numbers of PIMs in lungs from humans who died because of non-pulmonary causes. The cause for these unexpected findings cannot be determined. Because precise identification of PIMs can only be done using electron microscopy, immunohistology in this experiment detected septal macrophages which may represent the PIMs but also the macrophages in the interstitium or in the process of migration into the alveolar space. In addition, a reduction in alveolar macrophage numbers occurs following LPS inhalation in other species, possibly due to LPS-induced macrophage apoptosis (Bingisser *et al.* 1996; Pirie *et al.* 2001). Similar mechanisms may induce apoptosis in PIMs following long term exposure to LPS. However, this does not explain the fact that the non-diseased patients had a tendency of higher septal macrophage counts considering that under normal conditions human lungs are believed to be devoid of PIMs. The loss of normal pulmonary septal structures and fibrotic changes observed in hematoxylin and eosin stained sections from COPD/asthmatic patients indicates an exceedingly chronic process with no apparent inflammation. This can explain the low number of septal macrophages in some sections from diseased patients. In addition, the marked inflammation, edema and pulmonary haemorrhage observed in sections from control patients may explain the high number of septal macrophages in these sections. These observations may benefit from an increase in the sample size both from the COPD/asthmatic patients as well as from the control patients. The data obtained here might indicate, as opposed to previous reports, the occurrence of PIMs in normal human lung.

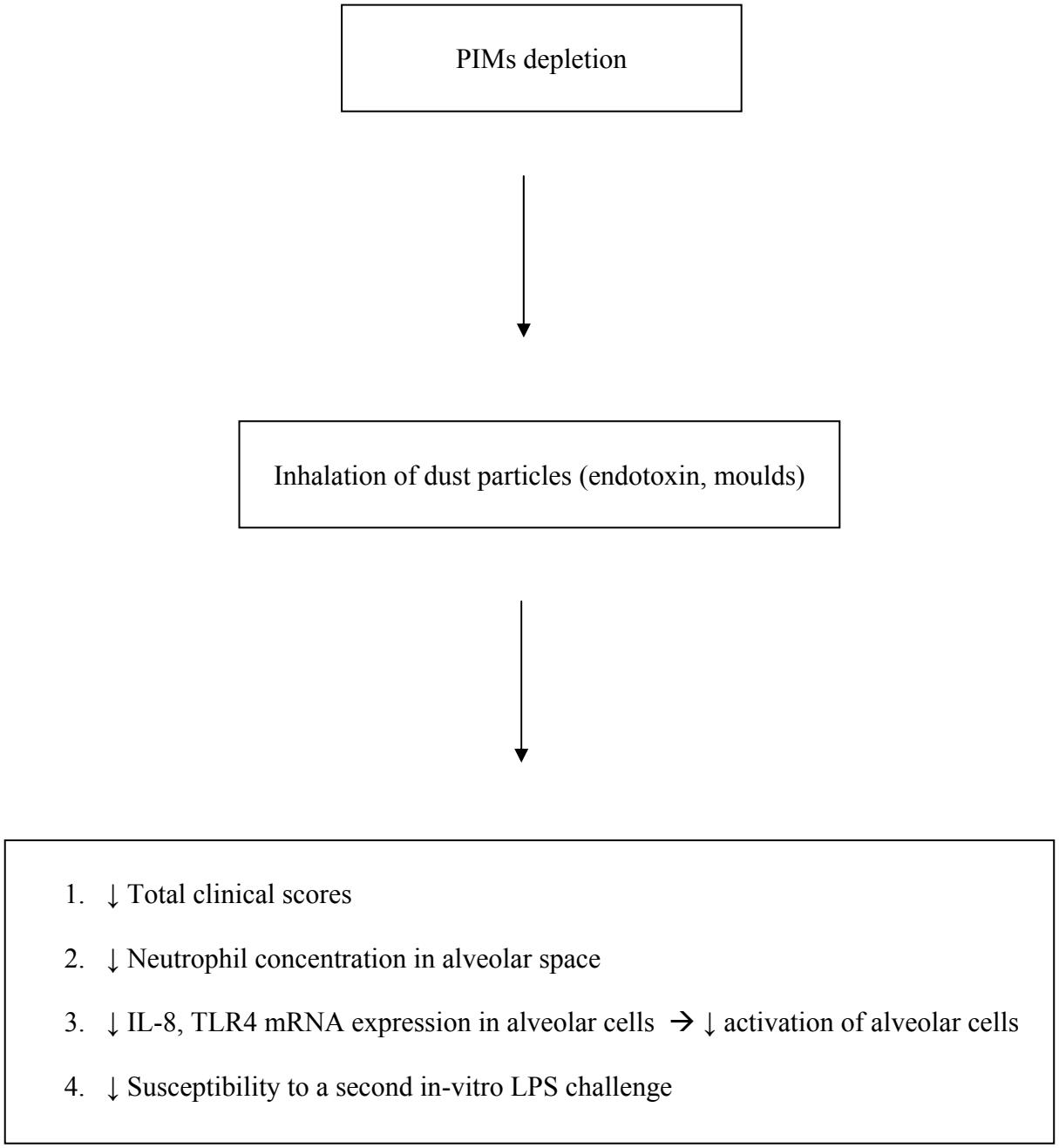

Figure 6.1. Outline of the major findings

7. SUMMARY

The first objective was to determine whether PIMs are involved in the clinical development of heaves in horses. The depletion of the PIMs when mares were fed with MH reduced the neutrophil concentration in the bronchoalveolar space and consequently reduced the development of clinical signs almost back to the control levels (cubes). This suggests an active role of the PIMs in the enhancement of heaves.

In order to better understand the involvement of PIMs in the pathogenesis of heaves, I examined the effect of PIM depletion on the activation state of BAL cells by determining the levels of three distinct pro-inflammatory molecules: TLR4 – a crucial receptor in the recognition of LPS; IL-8 – a neutrophilic chemoattractant; and TNFα – an acute phase pro-inflammatory cytokine. Interleukin-8 and TLR4 mRNA levels were both significantly decreased in BAL cells isolated from mares challenged with MH that received the GC treatment. The reduction in mRNA of these two pro-inflammatory molecules indicates an attenuation of BAL cell activation. In addition, consistent with the reduction of IL-8 mRNA expression fewer neutrophils had migrated to the bronchoavleolar space, and the reduction in the TLR4 mRNA expression would have lowered the susceptibility of the inflammatory cells to LPS and hence lowered the overall inflammatory response. Since mares in this study were susceptible to heaves and the BAL was performed after 7 days of MH exposure, the mares were probably at a later clinical stage and hence the TNFα, which is an acute phase pro-inflammatory cytokine, was not affected by the treatments. I also wanted to determine the effect of PIM depletion on a second *in vitro* challenge with LPS, which represents both an ongoing exposure to the endotoxin in the dust and a second challenge in heaves horses. The LPS *in vitro* challenge

was performed on BAL cells isolated from mares that underwent the different treatments (Cb, Cb-GC, MH, MH-GC). While the TNFα results were not conclusive, the second LPS challenge significantly increased the IL-8 mRNA expression in cells isolated from mares exposed to MH but not from mares exposed to MH and treated with GC. TLR4 did not seem to be affected by the second LPS challenge. However, similar to the IL-8 mRNA expression, in both LPS- and non-LPS-treated cells the TLR4 mRNA expression was lower in mares exposed to MH after treatment with GC as compared to the non-treated mares. Hence, depletion of PIMs reduced the activation state as well as the susceptibility of bronchoalveolar cells to a second challenge with LPS.

Lastly, I found a trend for reduced numbers of septal macrophages in lungs from humans suffering from non-infectious pulmonary diseases such as COPD or asthma compared to those who died due to non-pulmonary complications. If these septal macrophages are indeed PIMs, this finding is interesting as it indirectly indicates presence of PIMs in normal human lungs which is a controversial topic. These preliminary observations need to be expanded through an expanded sample size as well as additional techniques such as electron microscopy.

The following diagram summarizes the suggested involvement of PIMs in the development of an airborne pathology.

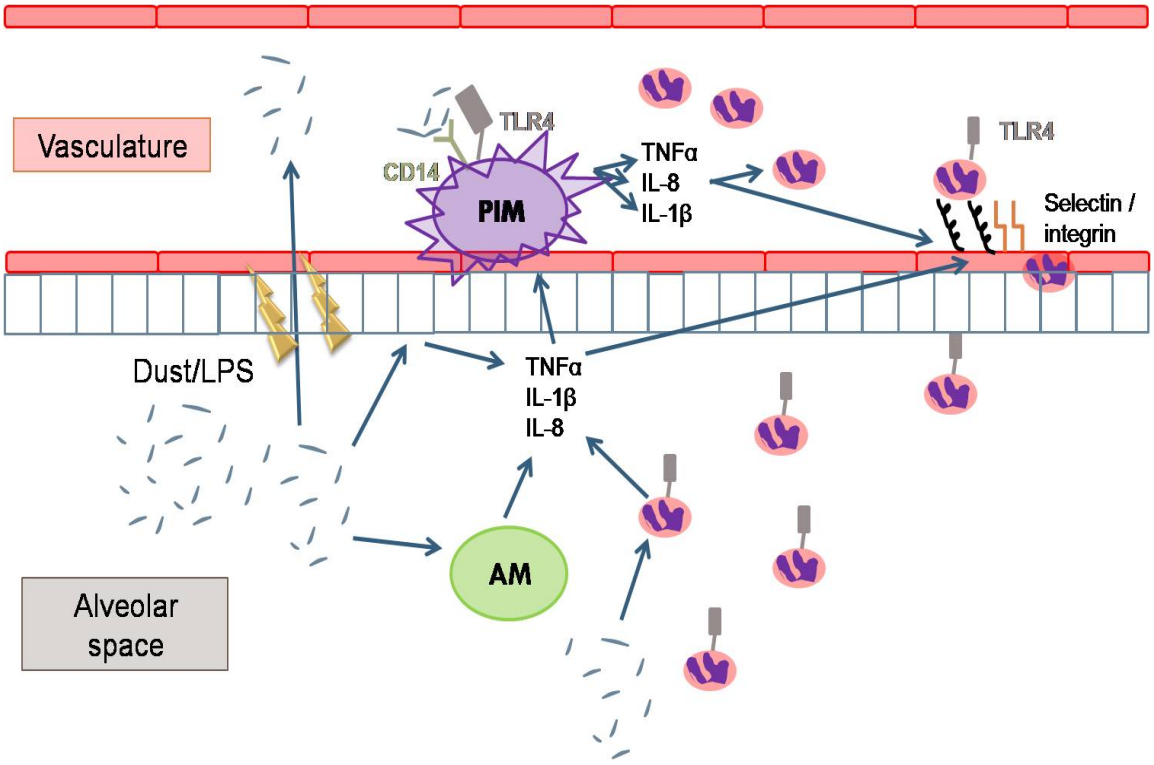

Figure 7.1. The suggested involvement of PIMs in the development of an airborne pathology. Inhaled dust and LPS activates the alveolar macrophages (AM) and the epithelial lining of the alveolar space. These activated cells produce proinflammatory mediators (i.e. TNFa, IL-1β, IL-8) which activate the PIMs and the endothelial cells to express adhesion molecules. LPS may also penetrate the vasculature though an injured blood-air barrier, bind to the CD14-TLR4 complex and further activate the PIMs. Activated PIMs produce inflammatory mediators which activate both circulating neutrophils as well as the endothelium. Activated neutrophils express high levels of TLR4, adhere to the endothelium and start migrating towards a chemotactic gradient into the alveolar space. At this stage there are high number of activated inflammatory cells in the alveolar space, due to the increase in the TLR4 expression these cells are more responsive to the inhaled LPS, which reactivates them, and they further contribute to the ongoing inflammatory process.

8. CONCLUSIONS

Gadolinium chloride treatment:

a. attenuates the development of clinical signs of heaves

b. attenuates the migration of inflammatory cells, especially of neutrophils to the alveolar space in the development of heaves

c. decreases the activation state of BAL cells, marked by a decrease in TLR4 and IL-8 mRNA, in the development of heaves

d. and reduces the activation of bronchoalveolar cells and their susceptibility to LPS.

Based on the data and above conclusions, the hypothesis that the depletion of PIMs in horses will attenuate the activation of airway inflammatory cells and reduce proinflammatory cytokines production in the development of heaves and in consequence will attenuate clinical signs of heaves and lower the response of BAL cells to a secondary challenge with LPS appears to be correct. Hence, PIMs play a major pro-inflammatory role in the development of an airborne pathology such as heaves in horses.

9. PROPOSED FUTURE RESEARCH

To further understand the role of PIMs in the pathogenesis of heaves, rather than in the development of heaves, and whether resident PIMs have a different role than recruited PIMs, it is important to pursue the clinical, cellular and molecular effects of the depletion of PIMs that have already been activated. Investigating the effect of alveolar macrophage depletion on the development of heaves will enhance our understanding on the role of alveolar macrophages in animals containing PIMs, the crosstalk between AM and PIMs, and whether AM function differently in animals containing PIMs than animals that are devoid of them. It would also be interesting to pursue the question of whether airborne LPS activates the PIMs directly or though secondary signalling molecules. Localization of LPS in the PIMs after airway exposure will indicate a direct activation of the PIMs by inhaled endotoxin. Lastly, the similarities between equine heaves and certain human pulmonary pathologies require further investigation on whether humans recruit PIMs under certain pathologic conditions. If indeed human recruit these specialized cells, studying their role in equine heaves can shed some light on the effect of PIMs in the development of such pathologies in human patients.

10. LIST OF REFERENCES

Aderem, A. (2001) Role of Toll-like receptors in inflammatory response in macrophages. *Crit Care Med* **29**, S16-18.

Aderem, A. and Ulevitch, R.J. (2000) Toll-like receptors in the induction of the innate immune response. *Nature* **406**, 782-787.

Adjers, K., Karjalainen, J., Pessi, T., Eklund, C. and Hurme, M. (2005) Epistatic effect of TLR4 and IL4 genes on the risk of asthma in females. *Int Arch Allergy Immunol* **138**, 251-256.

Ainsworth, D.M., Wagner, B., Franchini, M., Grunig, G., Erb, H.N. and Tan, J.Y. (2006) Time-dependent alterations in gene expression of interleukin-8 in the bronchial epithelium of horses with recurrent airway obstruction. *Am J Vet Res* **67**, 669-677.

Akira, S., Uematsu, S. and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124**, 783-801.

Andonegui, G., Bonder, C.S., Green, F., Mullaly, S.C., Zbytnuik, L., Raharjo, E. and Kubes, P. (2003) Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest* **111**, 1011-1020.

Antonelli, A., Bianchi, M., Crinelli, R., Gentilini, L. and Magnani, M. (2001) Modulation of ICAM-1 expression in ECV304 cells by macrophage-released cytokines. *Blood Cells Mol Dis* **27**, 978-991.

Art, T., Franck, T., Lekeux, P., de Moffarts, B., Couetil, L., Becker, M., Kohnen, S., Deby-Dupont, G. and Serteyn, D. (2006) Myeloperoxidase concentration in bronchoalveolar lavage fluid from healthy horses and those with recurrent airway obstruction. *Can J Vet Res* **70**, 291-296.

Art, T., Kirschvink, N., Smith, N. and Lekeux, P. (1999) Indices of oxidative stress in blood and pulmonary epithelium lining fluid in horses suffering from recurrent airway obstruction. *Equine Vet J* **31**, 397-401.

Atwal, O.S. and Minhas, K.J. (1992) In vivo interaction of cationised ferritin with the surface coat and endocytosis by pulmonary intravascular macrophages: a tracer kinetic study. *J Anat* **181 (Pt 2)**, 313-325.

Atwal, O.S. and Saldanha, K.A. (1985) Erythrophagocytosis in alveolar capillaries of goat lung: ultrastructural properties of blood monocytes. *Acta Anat (Basel)* **124**, 245-254.

Atwal, O.S., Singh, B., Staempfli, H. and Minhas, K. (1992) Presence of pulmonary intravascular macrophages in the equine lung: some structuro-functional properties. *Anat Rec* **234**, 530-540.

Bannenberg, G., Lundborg, M. and Johansson, A. (1995) Pulmonary macrophage function in systemic gadolinium chloride-pretreated rats. *Toxicol Lett* **80**, 105-107.

Baumgarten, G., Knuefermann, P., Wrigge, H., Putensen, C., Stapel, H., Fink, K., Meyer, R., Hoeft, A. and Grohe, C. (2006) Role of Toll-like receptor 4 for the pathogenesis of acute lung injury in Gram-negative sepsis. *Eur J Anaesthesiol* **23**, 1041-1048.

Berndt, A., Derksen, F.J., Venta, P.J., Ewart, S., Yuzbasiyan-Gurkan, V. and Robinson, N.E. (2007) Elevated amount of Toll-like receptor 4 mRNA in bronchial epithelial cells is associated with airway inflammation in horses with recurrent airway obstruction. *Am J Physiol Lung Cell Mol Physiol* **292**, L936-943.

Bertram, T.A. (1986) Intravascular macrophages in lungs of pigs infected with Haemophilus pleuropneumoniae. *Vet Pathol* **23**, 681-691.

Bingisser, R., Stey, C., Weller, M., Groscurth, P., Russi, E. and Frei, K. (1996) Apoptosis in human alveolar macrophages is induced by endotoxin and is modulated by cytokines. *Am J Respir Cell Mol Biol* **15**, 64-70.

Bowles, K.S., Beadle, R.E., Mouch, S., Pourciau, S.S., Littlefield-Chabaud, M.A., Le Blanc, C., Mistric, L., Fermaglich, D. and Horohov, D.W. (2002) A novel model for equine recurrent airway obstruction. *Vet Immunol Immunopathol* **87**, 385-389.

Brain, J.D., Molina, R.M., DeCamp, M.M. and Warner, A.E. (1999) Pulmonary intravascular macrophages: their contribution to the mononuclear phagocyte system in 13 species. *Am J Physiol* **276**, L146-154.

Brazil, T.J. (2001) The role of neutrophils in equine heaves. *Proceedings of the Second World Equine Airways Symposium, Scottland 2001*.

Brazil, T.J., Dagleish, M.P., McGorum, B.C., Dixon, P.M., Haslett, C. and Chilvers, E.R. (2005) Kinetics of pulmonary neutrophil recruitment and clearance in a natural and spontaneously resolving model of airway inflammation. *Clin Exp Allergy* **35**, 854-865.

Brikos, C. and O'Neill, L.A. (2008) Signalling of toll-like receptors. *Handb Exp Pharmacol*, 21-50.

Bureau, F., Bonizzi, G., Kirschvink, N., Delhalle, S., Desmecht, D., Merville, M.P., Bours, V. and Lekeux, P. (2000a) Correlation between nuclear factor-kappaB

activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. *Am J Respir Crit Care Med* **161**, 1314-1321.

Bureau, F., Delhalle, S., Bonizzi, G., Fievez, L., Dogne, S., Kirschvink, N., Vanderplasschen, A., Merville, M.P., Bours, V. and Lekeux, P. (2000b) Mechanisms of persistent NF-kappa B activity in the bronchi of an animal model of asthma. *J Immunol* **165**, 5822-5830.

Carrasco, L., Fernandez, A., Gomez Villamandos, J.C., Mozos, E., Mendez, A. and Jover, A. (1992) Kupffer cells and PIMs in acute experimental African swine fever. *Histol Histopathol* **7**, 421-425.

Carrasco, L., Nunez, A., Salguero, F.J., Diaz San Segundo, F., Sanchez-Cordon, P., Gomez-Villamandos, J.C. and Sierra, M.A. (2002) African swine fever: Expression of interleukin-1 alpha and tumour necrosis factor-alpha by pulmonary intravascular macrophages. *J Comp Pathol* **126**, 194-201.

Chang, S.W. and Ohara, N. (1994) Chronic biliary obstruction induces pulmonary intravascular phagocytosis and endotoxin sensitivity in rats. *J Clin Invest* **94**, 2009-2019.

Charavaryamath, C., Janardhan, K.S., Caldwell, S. and Singh, B. (2006) Pulmonary intravascular monocytes/macrophages in a rat model of sepsis. *Anat Rec A Discov Mol Cell Evol Biol* **288**, 1259-1271.

Charavaryamath, C., Janardhan, K.S., Townsend, H.G., Willson, P. and Singh, B. (2005) Multiple exposures to swine barn air induce lung inflammation and airway hyper-responsiveness. *Respir Res* **6**, 50.

Chitko-McKown, C.G. and Blecha, F. (1992) Pulmonary intravascular macrophages: a review of immune properties and functions. *Ann Rech Vet* **23**, 201-214.

Chitko-McKown, C.G., Chapes, S.K., Brown, R.E., Phillips, R.M., McKown, R.D. and Blecha, F. (1991) Porcine alveolar and pulmonary intravascular macrophages: comparison of immune functions. *J Leukoc Biol* **50**, 364-372.

Clements, J.M. and Pirie, R.S. (2007) Respirable dust concentrations in equine stables. Part 1: validation of equipment and effect of various management systems. *Res Vet Sci* **83**, 256-262.

Clere, J. and Heart, F. (1994) NIOSH Manual of Analytical Methods (NMAM), 0500, fourth edition [online]. The National Institute for Occupational Safety and Health. http://www.cdc.gov/NIOSH/nmam/pdfs/0500.pdf

Cordeau, M.E., Joubert, P., Dewachi, O., Hamid, Q. and Lavoie, J.P. (2004) IL-4, IL-5 and IFN-gamma mRNA expression in pulmonary lymphocytes in equine heaves. *Vet Immunol Immunopathol* **97**, 87-96.

Couetil, L.L., Rosenthal, F.S., DeNicola, D.B. and Chilcoat, C.D. (2001) Clinical signs, evaluation of bronchoalveolar lavage fluid, and assessment of pulmonary function in horses with inflammatory respiratory disease. *Am J Vet Res* **62**, 538-546.

Couetil, L.L. and Ward, M.P. (2003) Analysis of risk factors for recurrent airway obstruction in North American horses: 1,444 cases (1990-1999). *J Am Vet Med Assoc* **223**, 1645-1650.

Courouce-Malblanc, A., Fortier, G., Pronost, S., Siliart, B. and Brachet, G. (2007) Comparison of prednisolone and dexamethasone effects in the presence of environmental control in heaves-affected horses. *Vet J*.

Crocker, S.H., Eddy, D.O., Obenauf, R.N., Wismar, B.L. and Lowery, B.D. (1981) Bacteremia: host-specific lung clearance and pulmonary failure. *J Trauma* **21**, 215-220.

Davis, E. and Rush, B.R. (2002) Equine recurrent airway obstruction: pathogenesis, diagnosis, and patient management. *Vet Clin North Am Equine Pract* **18**, 453-467, vi.

Deaton, C.M., Marlin, D.J., Smith, N.C., Harris, P.A., Dagleish, M.P., Schroter, R.C. and Kelly, F.J. (2005) Effect of acute airway inflammation on the pulmonary antioxidant status. *Exp Lung Res* **31**, 653-670.

DeCamp, M.M., Warner, A.E., Molina, R.M. and Brain, J.D. (1992) Hepatic versus pulmonary uptake of particles injected into the portal circulation in sheep. Endotoxin escapes hepatic clearance causing pulmonary inflammation. *Am Rev Respir Dis* **146**, 224-231.

Dehring, D.J. and Wismar, B.L. (1989) Intravascular macrophages in pulmonary capillaries of humans. *Am Rev Respir Dis* **139**, 1027-1029.

Dixon, P.M., Railton, D.I. and McGorum, B.C. (1995) Equine pulmonary disease: a case control study of 300 referred cases. Part 2: Details of animals and of historical and clinical findings. *Equine Vet J* **27**, 422-427.

Eiznhamer, D.A., Flavin, M.T., Jesmok, G.J., Borgia, J.F., Nelson, D.J., Burhop, K.E. and Xu, Z.Q. (2004) Effective attenuation of endotoxin-induced acute lung injury by 2,3-diacetyloxybenzoic acid in two independent animal models. *Pulm Pharmacol Ther* **17**, 105-110.

Eyre, P. (1972) Equine pulmonary emphysema: a bronchopulmonary mould allergy. *Vet Rec* **91**, 134-140.

Feng, G., Liu, S., Wang, G.L. and Liu, G.J. (2008) Lidocaine attenuates lipopolysaccharide-induced acute lung injury through inhibiting NF-kappaB activation. *Pharmacology* **81**, 32-40.

Fernandez, S., Jose, P., Avdiushko, M.G., Kaplan, A.M. and Cohen, D.A. (2004) Inhibition of IL-10 receptor function in alveolar macrophages by Toll-like receptor agonists. *J Immunol* **172**, 2613-2620.

Franchini, M., Gilli, U., Akens, M.K., Fellenberg, R.V. and Bracher, V. (1998) The role of neutrophil chemotactic cytokines in the pathogenesis of equine chronic obstructive pulmonary disease (COPD). *Vet Immunol Immunopathol* **66**, 53-65.

Freeman, K.P., Roszel, J.F., McClure, J.M., Mannsman, R., Patton, P.E. and Naile, S. (1993) A review of cytological specimens from horses with and without clinical signs of respiratory disease. *Equine Vet J* **25**, 523-526.

Frevert, C.W. and Warner, A.E. (1992) Respiratory distress resulting from acute lung injury in the veterinary patient. *J Vet Intern Med* **6**, 154-165.

Fujihara, M., Muroi, M., Tanamoto, K., Suzuki, T., Azuma, H. and Ikeda, H. (2003) Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacol Ther* **100**, 171-194.

Gehr, P., Bachofen, M. and Weibel, E.R. (1978) The normal human lung: ultrastructure and morphometric estimation of diffusion capacity. *Respir Physiol* **32**, 121-140.

Giguere, S., Viel, L., Lee, E., MacKay, R.J., Hernandez, J. and Franchini, M. (2002) Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Vet Immunol Immunopathol* **85**, 147-158.

Gon, Y., Asai, Y., Hashimoto, S., Mizumura, K., Jibiki, I., Machino, T., Ra, C. and Horie, T. (2004) A20 inhibits toll-like receptor 2- and 4-mediated interleukin-8 synthesis in airway epithelial cells. *Am J Respir Cell Mol Biol* **31**, 330-336.

Horohov, D.W., Beadle, R.E., Mouch, S. and Pourciau, S.S. (2005) Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. *Vet Immunol Immunopathol* **108**, 237-245.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* **162**, 3749-3752.

Hulley, S.B., Cummings, S.R., Browner, W.S., Grady, D., Hearst, N. and Newman, T.B. (2001) *Designing Clinical Research*, second edn., Lippincott Williams and Wilkins, Philadelphia. p 169.

Imarisio, J.J. (1975) Liver scan showing intense lung uptake in neoplasia and infection. *J Nucl Med* **16**, 188-190.

Iversen, M., Kirychuk, S., Drost, H. and Jacobson, L. (2000) Human health effects of dust exposure in animal confinement buildings. *J Agric Saf Health* **6**, 283-288.

Jackson, C.A., Berney, C., Jefcoat, A.M. and Robinson, N.E. (2000) Environment and prednisone interactions in the treatment of recurrent airway obstruction (heaves). *Equine Vet J* **32**, 432-438.

Jeyaseelan, S., Chu, H.W., Young, S.K., Freeman, M.W. and Worthen, G.S. (2005) Distinct roles of pattern recognition receptors CD14 and Toll-like receptor 4 in acute lung injury. *Infect Immun* **73**, 1754-1763.

Kawai, T. and Akira, S. (2007) Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med*.

Kawasaki, T., Choudhry, M.A., Schwacha, M.G., Bland, K.I. and Chaudry, I.H. (2006) Lidocaine depresses splenocyte immune functions following trauma-hemorrhage in mice. *Am J Physiol Cell Physiol* **291**, C1049-1055.

Keyes, J.W., Jr., Wilson, G.A. and Quinonest, J.D. (1973) An evaluation of lung uptake of colloid during liver imaging. *J Nucl Med* **14**, 687-691.

Kirychuk, S., Senthilselvan, A., Dosman, J.A., Zhou, C., Barber, E.M., Rhodes, C.S. and Hurst, T.S. (1998) Predictors of longitudinal changes in pulmonary function among swine confinement workers. *Can Respir J* **5**, 472-478.

Kirychuk, S.P., Dosman, J.A., Reynolds, S.J., Willson, P., Senthilselvan, A., Feddes, J.J., Classen, H.L. and Guenter, W. (2006) Total dust and endotoxin in poultry operations: comparison between cage and floor housing and respiratory effects in workers. *J Occup Environ Med* **48**, 741-748.

Kleiber, C., Grunig, G., Jungi, T., Schmucker, N., Gerber, H., Davis, W.C. and Straub, R. (1999) Phenotypic analysis of bronchoalveolar lavage fluid lymphocytes in horses with chronic pulmonary disease. *Zentralbl Veterinarmed A* **46**, 177-184.

Kuebler, W.M., Parthasarathi, K., Wang, P.M. and Bhattacharya, J. (2000) A novel signaling mechanism between gas and blood compartments of the lung. *J Clin Invest* **105**, 905-913.

Laan, T.T., Bull, S., Pirie, R. and Fink-Gremmels, J. (2006) The role of alveolar macrophages in the pathogenesis of recurrent airway obstruction in horses. *J Vet Intern Med* **20**, 167-174.

Lavoie, J.P., Maghni, K., Desnoyers, M., Taha, R., Martin, J.G. and Hamid, Q.A. (2001) Neutrophilic airway inflammation in horses with heaves is characterized by a Th2-type cytokine profile. *Am J Respir Crit Care Med* **164**, 1410-1413.

Lawson, G.H., McPherson, E.A., Murphy, J.R., Nicholson, J.M., Wooding, P., Breeze, R.G. and Pirie, H.M. (1979) The presence of precipitating antibodies in the sera of horses with chronic obstructive pulmonary disease (COPD). *Equine Vet J* **11**, 172-176.

Leguillette, R. (2003) Recurrent airway obstruction--heaves. *Vet Clin North Am Equine Pract* **19**, 63-86, vi.

Liebers, V., Bruning, T. and Raulf-Heimsoth, M. (2006) Occupational endotoxin-exposure and possible health effects on humans. *Am J Ind Med* **49**, 474-491.

Longworth, K.E. (1997) The comparative biology of pulmonary intravascular macrophages. *Front Biosci* **2**, d232-241.

Longworth, K.E., Jarvis, K.A., Tyler, W.S., Steffey, E.P. and Staub, N.C. (1994) Pulmonary intravascular macrophages in horses and ponies. *Am J Vet Res* **55**, 382-388.

Longworth, K.E., Smith, B.L., Staub, N.C., Steffey, E.P. and Serikov, V.B. (1996) Use of detergent to prevent initial responses to endotoxin in horses. *Am J Vet Res* **57**, 1063-1066.

Longworth, K.E., Westgate, A.M., Grady, M.K., Westcott, J.Y. and Staub, N.C. (1992) Development of pulmonary intravascular macrophage function in newborn lambs. *J Appl Physiol* **73**, 2608-2615.

Madelin, T.M., Clarke, A.F. and Mair, T.S. (1991) Prevalence of serum precipitating antibodies in horses to fungal and thermophilic actinomycete antigens: effects of environmental challenge. *Equine Vet J* **23**, 247-252.

Marsik, C., Mayr, F., Cardona, F., Derhaschnig, U., Wagner, O.F. and Jilma, B. (2003) Endotoxaemia modulates Toll-like receptors on leucocytes in humans. *Br J Haematol* **121**, 653-656.

McGorum, B.C., Dixon, P.M. and Halliwell, R.E. (1993a) Phenotypic analysis of peripheral blood and bronchoalveolar lavage fluid lymphocytes in control and chronic obstructive pulmonary disease affected horses, before and after 'natural (hay and straw) challenges'. *Vet Immunol Immunopathol* **36**, 207-222.

McGorum, B.C., Dixon, P.M., Halliwell, R.E. and Irving, P. (1993b) Comparison of cellular and molecular components of bronchoalveolar lavage fluid harvested from different segments of the equine lung. *Res Vet Sci* **55**, 57-59.

McGorum, B.C., Ellison, J. and Cullen, R.T. (1998) Total and respirable airborne dust endotoxin concentrations in three equine management systems. *Equine Vet J* **30**, 430-434.

McPherson, E.A., Lawson, G.H., Murphy, J.R., Nicholson, J.M., Fraser, J.A., Breeze, R.G. and Pirie, H.M. (1978) Chronic obstructive pulmonary disease (COPD): identification of affected horses. *Equine Vet J* **10**, 47-53.

Michel, O., Ginanni, R., Duchateau, J., Vertongen, F., Le Bon, B. and Sergysels, R. (1991) Domestic endotoxin exposure and clinical severity of asthma. *Clin Exp Allergy* **21**, 441-448.

Michel, O., Kips, J., Duchateau, J., Vertongen, F., Robert, L., Collet, H., Pauwels, R. and Sergysels, R. (1996) Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* **154**, 1641-1646.

Mizgerd, J.P., Molina, R.M., Stearns, R.C., Brain, J.D. and Warner, A.E. (1996) Gadolinium induces macrophage apoptosis. *J Leukoc Biol* **59**, 189-195.

Monick, M.M., Yarovinsky, T.O., Powers, L.S., Butler, N.S., Carter, A.B., Gudmundsson, G. and Hunninghake, G.W. (2003) Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *J Biol Chem* **278**, 53035-53044.

Morris, G.E., Whyte, M.K., Martin, G.F., Jose, P.J., Dower, S.K. and Sabroe, I. (2005) Agonists of toll-like receptors 2 and 4 activate airway smooth muscle via mononuclear leukocytes. *Am J Respir Crit Care Med* **171**, 814-822.

Naylor, J.M., Clark, E.G. and Clayton, H.M. (1992) Chronic obstructive pulmonary disease: Usefulness of clinical signs, bronchoalveolar lavage, and lung biopsy as diagnostic and prognostic aids. *Can Vet J* **33**, 591-598.

Nevalainen, M., Raulo, S.M., Brazil, T.J., Pirie, R.S., Sorsa, T., McGorum, B.C. and Maisi, P. (2002) Inhalation of organic dusts and lipopolysaccharide increases gelatinolytic matrix metalloproteinases (MMPs) in the lungs of heaves horses. *Equine Vet J* **34**, 150-155.

Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K. and Akira, S. (2000) Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *J Immunol* **164**, 3476-3479.

Nys, M., Deby-Dupont, G., Habraken, Y., Legrand-Poels, S., Ledoux, D., Canivet, J.L., Damas, P. and Lamy, M. (2002) Bronchoalveolar lavage fluids of patients with lung injury activate the transcription factor nuclear factor-kappaB in an alveolar cell line. *Clin Sci (Lond)* **103**, 577-585.

Ordonez, C.L., Shaughnessy, T.E., Matthay, M.A. and Fahy, J.V. (2000) Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: Clinical and biologic significance. *Am J Respir Crit Care Med* **161**, 1185-1190.

Parbhakar, O.P., Duke, T., Townsend, H.G. and Singh, B. (2004) Immunophenotypic characterization and depletion of pulmonary intravascular macrophages of horses. *Vet Res* **35**, 39-51.

Parbhakar, O.P., Duke, T., Townsend, H.G. and Singh, B. (2005) Depletion of pulmonary intravascular macrophages partially inhibits lipopolysaccharide-induced lung inflammation in horses. *Vet Res* **36**, 557-569.

Pirie, R.S., Collie, D.D., Dixon, P.M. and McGorum, B.C. (2003a) Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma). *Clin Exp Allergy* **33**, 676-683.

Pirie, R.S., Dixon, P.M., Collie, D.D. and McGorum, B.C. (2001) Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses. *Equine Vet J* **33**, 311-318.

Pirie, R.S., Dixon, P.M. and McGorum, B.C. (2002) Evaluation of nebulised hay dust suspensions (HDS) for the diagnosis and investigation of heaves. 3: Effect of fractionation of HDS. *Equine Vet J* **34**, 343-347.

Pirie, R.S., Dixon, P.M. and McGorum, B.C. (2003b) Endotoxin contamination contributes to the pulmonary inflammatory and functional response to Aspergillus fumigatus extract inhalation in heaves horses. *Clin Exp Allergy* **33**, 1289-1296.

Raulo, S.M., Sorsa, T., Tervahartiala, T., Pirila, E. and Maisi, P. (2001) MMP-9 as a marker of inflammation in tracheal epithelial lining fluid (TELF) and in bronchoalveolar fluid (BALF) of COPD horses. *Equine Vet J* **33**, 128-136.

Rizzo, M.C., Naspitz, C.K., Fernandez-Caldas, E., Lockey, R.F., Mimica, I. and Sole, D. (1997) Endotoxin exposure and symptoms in asthmatic children. *Pediatr Allergy Immunol* **8**, 121-126.

Robinson, N.E. (2001) International Workshop on Equine Chronic Airway Disease. Michigan State University 16-18 June 2000. *Equine Vet J* **33**, 5-19.

Robinson, N.E., Berney, C., Eberhart, S., deFeijter-Rupp, H.L., Jefcoat, A.M., Cornelisse, C.J., Gerber, V.M. and Derksen, F.J. (2003) Coughing, mucus accumulation, airway obstruction, and airway inflammation in control horses and horses affected with recurrent airway obstruction. *Am J Vet Res* **64**, 550-557.

Robinson, N.E., Derksen, F.J., Olszewski, M.A. and Buechner-Maxwell, V.A. (1996) The pathogenesis of chronic obstructive pulmonary disease of horses. *Br Vet J* **152**, 283-306.

Robinson, N.E., Karmaus, W., Holcombe, S.J., Carr, E.A. and Derksen, F.J. (2006) Airway inflammation in Michigan pleasure horses: prevalence and risk factors. *Equine Vet J* **38**, 293-299.

Rogers, R.A., Tasat, D.R., Warner, A.E. and Brain, J.D. (1994) Quantitative recovery of pulmonary intravascular macrophages from sheep lungs. *J Leukoc Biol* **56**, 692-701.

Rush, B.R., Flaminio, M.J., Matson, C.J., Hakala, J.E. and Shuman, W. (1998a) Cytologic evaluation of bronchoalveolar lavage fluid from horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am J Vet Res* **59**, 1033-1038.

Rush, B.R., Raub, E.S., Rhoads, W.S., Flaminio, M.J., Matson, C.J., Hakala, J.E. and Gillespie, J.R. (1998b) Pulmonary function in horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *Am J Vet Res* **59**, 1039-1043.

Rushton, L. (2007) Occupational causes of chronic obstructive pulmonary disease. *Rev Environ Health* **22**, 195-212.

Rybicka, K., Daly, B.D., Migliore, J.J. and Norman, J.C. (1974) Ultrastructure of pulmonary alveoli of the calf. *Am J Vet Res* **35**, 213-222.

Rylander, R. and Bergstrom, R. (1993) Bronchial reactivity among cotton workers in relation to dust and endotoxin exposure. *Ann Occup Hyg* **37**, 57-63.

Sabroe, I., Jones, E.C., Usher, L.R., Whyte, M.K. and Dower, S.K. (2002) Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol* **168**, 4701-4710.

Sabroe, I., Prince, L.R., Jones, E.C., Horsburgh, M.J., Foster, S.J., Vogel, S.N., Dower, S.K. and Whyte, M.K. (2003) Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. *J Immunol* **170**, 5268-5275.

Sasse, H.H.L. (1971) Some Pulmonary Function Test in Horses: An Aid to Early Diagnosis of Chronic Obstructive Pulmonary Disease ('heaves') in Horses. PhD Thesis, University of Utrecht, The Netherlands.

Schmallenbach, K.H., Rahman, I., Sasse, H.H., Dixon, P.M., Halliwell, R.E., McGorum, B.C., Crameri, R. and Miller, H.R. (1998) Studies on pulmonary and systemic Aspergillus fumigatus-specific IgE and IgG antibodies in horses affected with chronic obstructive pulmonary disease (COPD). *Vet Immunol Immunopathol* **66**, 245-256.

Schwartz, D.A. (2001) The role of TLR4 in endotoxin responsiveness in humans. *J Endotoxin Res* **7**, 389-393.

Sierra, M.A., Carrasco, L., Gomez-Villamandos, J.C., Martin de las Mulas, J., Mendez, A. and Jover, A. (1990) Pulmonary intravascular macrophages in lungs of pigs inoculated with African swine fever virus of differing virulence. *J Comp Pathol* **102**, 323-334.

Simonen-Jokinen, T., Pirie, R.S., McGorum, B.C. and Maisi, P. (2005) Effect of composition and different fractions of hay dust suspension on inflammation in lungs of heaves-affected horses: MMP-9 and MMP-2 as indicators of tissue destruction. *Equine Vet J* **37**, 412-417.

Singh, B., Atwal, O.S. and Minhas, K. (1995) Surface coat of sheep pulmonary intravascular macrophages is reconstituted following brefeldin A-mediated endocytosis. *J Submicrosc Cytol Pathol* **27**, 235-249.

Singh, B. and de la Concha-Bermejillo, A. (1998) Gadolinium chloride removes pulmonary intravascular macrophages and curtails the degree of ovine lentivirus-induced lymphoid interstitial pneumonia. *Int J Exp Pathol* **79**, 151-162.

Singh, B., Minhas, K.J. and Atwal, O.S. (1994) Ultracytochemical study of multiple dose effect of monastral blue uptake by equine pulmonary intravascular macrophages (PIMs). *J Submicrosc Cytol Pathol* **26**, 235-243.

Singh, B., Pearce, J.W., Gamage, L.N., Janardhan, K. and Caldwell, S. (2004) Depletion of pulmonary intravascular macrophages inhibits acute lung inflammation. *Am J Physiol Lung Cell Mol Physiol* **286**, L363-372.

Singh Suri, S., Janardhan, K.S., Parbhakar, O., Caldwell, S., Appleyard, G. and Singh, B. (2006) Expression of toll-like receptor 4 and 2 in horse lungs. *Vet Res* **37**, 541-551.

Smid, T., Heederik, D., Houba, R. and Quanjer, P.H. (1994) Dust- and endotoxin-related acute lung function changes and work-related symptoms in workers in the animal feed industry. *Am J Ind Med* **25**, 877-888.

Sone, Y., Nicolaysen, A. and Staub, N.C., Sr. (1997) Effect of particles on sheep lung hemodynamics parallels depletion and recovery of intravascular macrophages. *J Appl Physiol* **83**, 1499-1507.

Sone, Y., Serikov, V.B. and Staub, N.C., Sr. (1999) Intravascular macrophage depletion attenuates endotoxin lung injury in anesthetized sheep. *J Appl Physiol* **87**, 1354-1359.

Springer, T.A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301-314.

Staub, N.C. (1994) Pulmonary intravascular macrophages. *Annu Rev Physiol* **56**, 47-67.

Staub, N.C., Sr., Longworth, K.E., Serikov, V., Jerome, E.H. and Elsasser, T. (2001) Detergent inhibits 70-90% of responses to intravenous endotoxin in awake sheep. *J Appl Physiol* **90**, 1788-1797.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**, 443-451.

Tesarowski, D.B., Viel, L. and McDonell, W.N. (1996) Pulmonary function measurements during repeated environmental challenge of horses with recurrent airway obstruction (heaves). *Am J Vet Res* **57**, 1214-1219.

Togbe, D., Schnyder-Candrian, S., Schnyder, B., Doz, E., Noulin, N., Janot, L., Secher, T., Gasse, P., Lima, C., Coelho, F.R., Vasseur, V., Erard, F., Ryffel, B., Couillin, I. and Moser, R. (2007) Toll-like receptor and tumour necrosis factor dependent endotoxin-induced acute lung injury. *Int J Exp Pathol* **88**, 387-391.

Tremblay, G.M., Ferland, C., Lapointe, J.M., Vrins, A., Lavoie, J.P. and Cormier, Y. (1993) Effect of stabling on bronchoalveolar cells obtained from normal and COPD horses. *Equine Vet J* **25**, 194-197.

Turlej, R.K., Fievez, L., Sandersen, C.F., Dogne, S., Kirschvink, N., Lekeux, P. and Bureau, F. (2001) Enhanced survival of lung granulocytes in an animal model of asthma: evidence for a role of GM-CSF activated STAT5 signalling pathway. *Thorax* **56**, 696-702.

Uematsu, S. and Akira, S. (2006) Toll-like receptors and innate immunity. *J Mol Med* **84**, 712-725.

Vandenput, S., Votion, D., Duvivier, D.H., Van Erck, E., Anciaux, N., Art, T. and Lekeux, P. (1998) Effect of a set stabled environmental control on pulmonary function and airway reactivity of COPD affected horses. *Vet J* **155**, 189-195.

Vogelzang, P.F., van der Gulden, J.W., Folgering, H., Kolk, J.J., Heederik, D., Preller, L., Tielen, M.J. and van Schayck, C.P. (1998) Endotoxin exposure as a major determinant of lung function decline in pig farmers. *Am J Respir Crit Care Med* **157**, 15-18.

Vrins, A., Doucet, M. and Nunez-Ochoa, L. (1991) A retrospective study of bronchoalveolar lavage cytology in horses with clinical findings of small airway disease. *Zentralbl Veterinarmed A* **38**, 472-479.

Wang, H.M., Bodenstein, M. and Markstaller, K. (2008) Overview of the pathology of three widely used animal models of acute lung injury. *Eur Surg Res* **40**, 305-316.

Warner, A.E. (1996) Pulmonary intravascular macrophages. Role in acute lung injury. *Clin Chest Med* **17**, 125-135.

Warner, A.E., Barry, B.E. and Brain, J.D. (1986) Pulmonary intravascular macrophages in sheep. Morphology and function of a novel constituent of the mononuclear phagocyte system. *Lab Invest* **55**, 276-288.

Warner, A.E., Molina, R.M. and Brain, J.D. (1987) Uptake of bloodborne bacteria by pulmonary intravascular macrophages and consequent inflammatory responses in sheep. *Am Rev Respir Dis* **136**, 683-690.

Wassef, A., Janardhan, K., Pearce, J.W. and Singh, B. (2004) Toll-like receptor 4 in normal and inflamed lungs and other organs of pig, dog and cattle. *Histol Histopathol* **19**, 1201-1208.

Watson, J.L., Stott, J.L., Blanchard, M.T., Lavoie, J.P., Wilson, W.D., Gershwin, L.J. and Wilson, D.W. (1997) Phenotypic characterization of lymphocyte subpopulations in horses affected with chronic obstructive pulmonary disease and in normal controls. *Vet Pathol* **34**, 108-116.

Werner, M., Topp, R., Wimmer, K., Richter, K., Bischof, W., Wjst, M. and Heinrich, J. (2003) TLR4 gene variants modify endotoxin effects on asthma. *J Allergy Clin Immunol* **112**, 323-330.

Wheeldon, E.B. and Hansen-Flaschen, J.H. (1986) Intravascular macrophages in the sheep lung. *J Leukoc Biol* **40**, 657-661.

Whiteley, L.O., Maheswaran, S.K., Weiss, D.J. and Ames, T.R. (1990) Immunohistochemical localization of Pasteurella haemolytica A1-derived endotoxin, leukotoxin, and capsular polysaccharide in experimental bovine Pasteurella pneumonia. *Vet Pathol* **27**, 150-161.

Whiteley, L.O., Maheswaran, S.K., Weiss, D.J. and Ames, T.R. (1991) Alterations in pulmonary morphology and peripheral coagulation profiles caused by intratracheal inoculation of live and ultraviolet light-killed Pasteurella haemolytica A1 in calves. *Vet Pathol* **28**, 275-285.

Winkler, G.C. (1988) Pulmonary intravascular macrophages in domestic animal species: review of structural and functional properties. *Am J Anat* **181**, 217-234.

Winkler, G.C. and Cheville, N.F. (1985) Monocytic origin and postnatal mitosis of intravascular macrophages in the porcine lung. *J Leukoc Biol* **38**, 471-480.

Winkler, G.C. and Cheville, N.F. (1987) Postnatal colonization of porcine lung capillaries by intravascular macrophages: an ultrastructural, morphometric analysis. *Microvasc Res* **33**, 224-232.

Woods, P.S., Robinson, N.E., Swanson, M.C., Reed, C.E., Broadstone, R.V. and Derksen, F.J. (1993) Airborne dust and aeroallergen concentration in a horse stable under two different management systems. *Equine Vet J* **25**, 208-213.

Zeltner, T.B., Caduff, J.H., Gehr, P., Pfenninger, J. and Burri, P.H. (1987) The postnatal development and growth of the human lung. I. Morphometry. *Respir Physiol* **67**, 247-267.